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IN VIVO STUDIES ON THE TOXIC EFFECTS OF ORELLANINE: SEARCH FOR EFFICIENT ANTIDOTES

Dissertação de 2º Ciclo de Estudos Conducente ao Grau de Mestre em Toxicologia Analítica, Clínica e Forense na Faculdade de Farmácia da Universidade do Porto

Trabalho realizado sob a orientação do Professor Doutor Ricardo Jorge Dinis Oliveira e coorientação da Professora Doutora Carolina Amorim e Mestre Emanuele Amorim Alves

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DECLARATIONS

1. É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA DISSERTAÇÃO APENAS PARA EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE COMPROMETE.

PUBLICATIONS AND POSTER COMMUNICATIONS

The results presented in this thesis led to the following works:

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ABSTRACT

Orellanine (OR) is a nephrotoxic toxin produced by some mushroom species of the *Cortinarius* genus, typically found in Europe and North America. The nephrotoxicity of *Cortinarius orellanus* is well known and was first recognized in the 1950s when this mushroom was identified as the cause of a mass poisoning in Poland. This toxin induces an acute renal failure which can be very severe or even irreversible and fatal. The mechanism of toxicity is not yet fully understood, but it has been shown that the toxin inhibits the synthesis of proteins, RNA and DNA, and generates reactive oxygen species (ROS) and subsequent oxidative stress. Chemically, orellanine resembles the bipyridyl herbicides diquat and paraquat (PQ). PQ toxicity is specially severe in the lungs both due to its accumulation, against a concentration gradient, through the highly developed polyamine uptake system, and capacity to continuously generate ROS through a redox cycle involving PQ radicals and oxygen, leading to oxidative stress-related cell death and inflammation. In prior studies, it was demonstrated that therapeutic regimen with lysine acetylsalicylate (LAS) has a great potential to be considered in the hospital treatment of PQ poisonings. Indeed, it was observed a full survival in rats treated with PQ+LAS and the structural alterations in the lung, kidney, liver and jejune were considerably attenuated, specially concerning to collagen deposition.

Due to the high toxic potential of OR and considering the absence of a specific antidote for this poisoning, the aim of this dissertation was to evaluate the potential protective effect of LAS.

Animals were divided in four groups that were treated as follows: (1) control group, animals were subjected to two 0.9% saline solution administrations (0 and 4 h); (2) OR group, animals were exposed to one dose of OR (15 mg/kg) followed by one 0.9% saline solution 4 h after OR administration; (3) LAS group, animals were exposed to a 0.9% saline solution followed by one dose of LAS (200 mg/kg) 4 h after saline administration; and (4) OR + LAS group, animals were exposed to one dose of OR (15 mg/kg) followed by one dose of LAS (200 mg/kg) 4 h later. Twenty-four hours after the last administration, all animals were anesthetized with xylazine/ketamine (10 and 100 mg/kg, respectively). All administrations were given intraperitoneally. Blood, urine and solid tissue samples (liver, kidneys, lungs, heart and spleen) were collected for subsequent biochemical analysis.

A statistically significant increase of uric acid (UA) in OR group (1.14±0.39 mg/dL, $p<0.05$), when compared with control group (0.20±0.07 mg/dL), was registered. This increased UA was not observed in OR+LAS group (0.30±0.12 mg/dL). The increased UA in OR group can either be related to a possible kidney damage or to an increased

catabolism of purines. Indeed, OR caused notorious kidney damage, with tubular ectasia, tubular proteinosis, and epithelial cells of the affected tubules exhibited marked cytoplasmic degenerative changes or necrosis and apoptosis. The histological kidney findings also showed that LAS did not revert the damage caused by OR, being that, the volume of the affected tubules was apparently higher in the rats of OR+LAS group. The liver was also shown to be an organ affected by OR poisoning. Mild lobular inflammatory activity, parenchymal damage, hemorrhage and absence of viable hepatocytes were evident in animals treated with OR. The animals exposed to OR+LAS had livers with an apparent higher volume of lesions. The oxidative stress biomarker, GSSG, was markedly increased in OR+LAS group (0.76±0.24 and 14.93±1.60 U/L, $p<0.01$) when compared to control group (0.09±0.01 and 19.52±2.31 U/L) for kidney and liver, respectively. Accordingly, GSH/GSSG ratio suffered a significant reduction (0.58±0.23 ($p<0.05$) and 1.138±0.3135 ($p<0.0001$)) compared to the control group (13.00±2.59 and 11.28±1.89).

Taken together, these results demonstrate that LAS do not confer a protection against orellanine induced-toxicity and, in some organs, it seems to worsen injury.

Keywords: Orellanine; lysine acetylsalicylate; kidney; liver; injury

RESUMO

A orelanina (OR) é uma toxina nefrotóxica produzida por algumas espécies de cogumelos do género *Cortinarius*, que normalmente são encontrados na Europa e na América do Norte. A nefrotoxicidade do *Cortinarius orellanus* é bem conhecida desde 1950, quando este cogumelo foi identificado como causa de um elevado número de intoxicações na Polónia. Esta toxina induz uma insuficiência renal aguda que pode ser muito grave ou mesmo irreversível e fatal. O mecanismo de toxicidade ainda não é totalmente compreendido, mas tem sido demostrado que esta toxina inibe a síntese de proteínas, RNA e DNA, gera espécies reativas de oxigénio (ROS) e consequente *stress* oxidativo. Quimicamente, a OR assemelha-se aos herbicidas bipiridílios diquato e paraquato (PQ). A toxicidade do PQ é essencialmente grave nos pulmões devido quer à sua acumulação, contra um gradiente de concentração, por meio de um sistema de captação de poliaminas, quer à sua capacidade para gerar continuamente ROS através de um ciclo redox que envolve radicas PQ e oxigénio, levando a uma situação de *stress* oxidativo, morte celular e inflamação. Em estudos anteriores, foi demonstrado que um regime terapêutico com acetilsalicilato de lisina (LAS) apresenta grande potencial para ser utilizado no tratamento hospitalar da intoxicação por PQ. De facto, foi observada uma total sobrevivência em todos os ratos tratados com PQ+LAS, sendo que as alterações estruturais no pulmão, rim, fígado e jejuno foram consideravelmente atenuadas, especialmente em relação à deposição de colagénio.

Devido ao alto potencial tóxico da OR e considerando a inexistência de um antídoto específico para esta intoxicação, o objetivo deste trabalho foi avaliar o potencial efeito protetor do LAS.

Os animais foram divididos em quatro grupos, tratados de acordo com o seguinte: (1) grupo controlo, os animais foram tratados às 0 e 4 horas com solução de soro fisiológico a 0.9%; (2) grupo OR, os animais foram expostos a uma dose de OR (15 mg/kg), seguida por uma dose de soro fisiológico a 0.9% 4h após a administração de OR; (3) grupo LAS, os animais foram tratados com soro fisiológico a 0.9%, seguido de uma dose de LAS (200 mg/kg) 4h após a administração do soro; (4) grupo OR+LAS, os animais foram submetidos a uma dose de OR (15 mg/kg) seguida por uma dose de LAS (200 mg/kg) 4h após. 24 horas após a última administração, todos os animais foram anestesiados com xilazina e ketamina (10 e 100 mg/kg, respetivamente). Todas as administrações foram feitas por via intraperitoneal. Amostras de sangue, urina e tecidos (fígado, rins, pulmões, coração e baço) foram colhidas para subsequente análise bioquímica.

Foi observado um aumento estatisticamente significativo de ácido úrico (UA) no grupo OR (1.14±0,39 mg/dL, *p<0.05*), quando comparado com o grupo controlo (0,20±0,07 mg/dL). Este aumento não foi observado no grupo OR+LAS (0,30±0,12 mg/dL). O aumento de UA no grupo OR pode estar relacionado com uma possível lesão renal ou com o aumento do catabolismo das purinas. De facto, a OR causou danos notórios nos rins, como ectasia tubular, proteinose tubular e as células epiteliais dos túbulos afetados exibiram alterações citoplasmáticas marcadas e degenerativas ou necrose e apoptose. Estes achados histológicos renais mostraram também que o LAS não reverteu o dano causado pela OR, sendo que o volume dos túbulos afetados foi aparentemente mais elevado nos ratos do grupo OR+LAS. O fígado também demonstrou ser um órgão afetado pela intoxicação com OR. Atividade inflamatória lobular, lesão parenquimatosa, hemorragia e ausência de hepatócitos viáveis foram evidentes nos animais tratados com OR. Os animais expostos a OR+LAS apresentaram fígados com um maior volume aparente de lesões. O biomarcador de stress oxidativo, GSSG, mostrou-se significativamente aumentado no grupo OR+LAS (0,76±0,24 e 14,93±1.60 U/L *p<0.01*) quando comparado com o grupo controlo (0,09±0,01 e 19,52±2.31 U/L) para o rim e fígado, respetivamente. Em conformidade, o rácio GSH/GSSG sofreu uma redução significativa (0,58±0,23 (*p<0.05)* e 1,14±0,31 (*p<0.0001)*) em comparação com o grupo controlo (13.00±2.59 e 11,28±1.89).

No seu conjunto, estes resultados demonstram que o LAS não confere proteção contra a toxicidade induzida pela OR e que, em alguns órgãos, parece agravar as lesões.

Palavras-chave: Orelanina; acetilsalicilato de lisina; rim; fígado; lesão.

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LIST OF ABBREVIATIONS

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AST – Aspartate aminotransferase

BUN – Urea nitrogen

***C.* –** *Cortinarius*

C_3H_8O – Isopropyl alcohol

Ca – Calcium

CA – Central artery

CAT – Catalase

$CHCl_3$ – Cloroformic

Chol – Cholesterol

CIAV – Antipoison information center (Centro de Informação Antivenenos)

CK – Creatinine kinase

CK-MB – Creatinine kinase MB

CNT – *Cortinarius* nephron toxicity prognostic index

Ct – Threshold cycle

CV – Central vein

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DT – Distal tubules

DTNB – 5,5'-dithiobis-2-nitrobenzoic acid

EDTA – Ethylenediaminetetraacetic acid

$FeCL_3$:$6H_2O$ – Ferric chloride hexahydrate

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GGT – γ-glutamyl-transpeptidase

GPX3 – Glutathione peroxidase

GSH – Reduced glutathione

GSHt – Total glutathione

GSSG – Oxidized glutathione

H_2O_2 – Hydrogen peroxide

HCL – Hydrochloric acid

$HClO_4$ – Perchloric acid

HDL – High-density lipoprotein

HPLC – High-performance liquid chromatography

IP – Intraperitoneal

KH_2PO_4 – Potassium dihydrogen phosphate

$KHCO_3$ – Potassium bicarbonate

LAS – Lysine acetylsalicylate

LD_{50} – Lethal dose

LDH – Lactate dehydrogenase

LDL – Low density lipoprotein

MDA – Malondialdehyde

Meg – Megakaryocyte

Mg – Magnesium

mRNA – Messenger RNA

Na_2HPO_4 – Disodium phosphate

NaCl – Sodium chloride

NADPH – Nicotinamide adenine dinucleotide phosphate

NAG – N-acetyl-β-glucosaminidase

NaH_2PO_4 – Monosodium phosphate

NaOH – Sodium hydroxide

O_2 – Oxygen

OR – Orellanine

PchE – Pseudocholinesterase

Phos – Phosphate

PO – *Per os*

PQ – Paraquat

PT – Proximal tubules

qPCR – Quantitative real-time polymerase chain reaction

R_f – Retention factor

RNA – Ribonucleic acid

RNAP I – RNA polymerase I

RNAP II – RNA polymerase II

ROS – Reactive oxygen species

RP – Red pulp

SC – Subcutaneous

SEM – Standard error of the mean

SGOT – Serum glutamate-oxaloacetate-transaminase

SGPT – Serum glutamate-pyruvate-transaminase

SOD – Superoxide dismutase

SOD1 – Cytoplasmic CuZn-SOD

TBA – 2-thiobarbituric acid

TG – Triglycerides

TP – Total protein

UA – Uric acid

WP – White pulp

β-actin – Beta actin

Part I

Introduction

1. INTRODUCTION

1.1 Mushrooms poisoning

Mushrooms are ubiquitous in nature. They are an important source of nutrition, are high in protein and essential amino acids and tend to be fat free, cholesterol free, and low in calories. However, certain varieties contain chemicals that can be highly toxic to humans (Berger and Guss, 2005). There are about 5000 mushrooms species, of which approximately 50 - 100 are known to be poisonous to humans, whereas only 200 to 300 varieties have been clearly established to be safely edible (Barbato, 1993; Brent and Kulig, 1998; Bryson, 1996; Ellenhorn and Barceloux, 1997).

Mushroom poisoning is a global phenomenon and can be a source of major mortality and morbidity. This poisoning in humans has been described since time immemorial, which has been witnessed by ancient writings like “Rigveda” (at least 3500 B.C.) and “Atharvaveda” (at least 1500 B. C.). The first written record about a fungus is the death from fungal poisoning, of a mother, daughter and two full grown sons, an event, which Euripides (456–450 B.C.) commemorated by an epigram (kumar Jha and Tripathi). Mushroom poisoning is usually the result of ingestion of wild mushrooms due to misidentification of a toxic mushroom as an edible species. More than 95% of mushroom poisoning incidences around the world occur due to misidentification (Duffy, 2008). Recently, incidence of mushroom poisoning is increasing as a result of the increasing popularity of wild mushroom consumption. The incidence is more frequent in Western Europe with 50-100 fatal cases reported annually. Likewise, the incidence is 0.005% in USA and 0.05% in Iran (Kavalci et al., 2010).

Toxic mushrooms can be grouped based on their toxic components: Amanitins (Cyclopeptides), Gyromitrin (Monomethylhydrazine), Orellanine (OR), Muscarine, Ibotenic acid and Muscimol, Coprine, *Psilocybin* (psilocin) and Gastrointestinal irritants (Raut et al., 2014). Diaz *et al* (2005) has reviewed and established the classification of mushroom poisoning based on the time of presentation and target organ systemic toxicity. With respect to the time of presentation, mushroom poisoning is categorized as early onset (<6 hours), late onset (6-24 hours), or delayed onset (>1 day). Early-onset toxicities include several neurotoxic, gastrointestinal and allergic syndromes. Late-onset toxicities include hepatotoxic, accelerated nephrotoxic and erythromelalgia syndromes. Delayed-onset toxicities include delayed nephrotoxic, delayed neurotoxic and rhabdomyolytic syndromes (Diaz, 2005).

Accurate estimates of worldwide poison by OR-containing mushrooms are difficult to establish due to lack of case reporting in hospital emergency rooms. In Portugal there is only one retrospective analysis of 93 cases of mushroom poisoning admitted in ten Portuguese hospitals between 1990 and 2008. Of those poisonings 63.4% were attributed to amatoxins-containing mushrooms and 31.7% were attributed to gastrointestinal irritants-containing mushrooms (Brandão et al., 2011). In 2015 and 2014 were reported to the Poison Information Centers (CIAV) 31 and 49 cases for mushroom intoxications, respectively (data is required CIAV). The Portuguese population is essentially mycophobic, and the picking and consumption of wild mushrooms is generally restricted to rural areas, and to a small number of species. This fact can limit cases of poisoning to a typology that is easily recognizable locally (Brandão et al., 2011). In USA, a total of 6275, 6818, 6600, 6575 and 6474 mushrooms intoxications were reported to the national poison data system of the American Association of Poison Control Centers in 2010, 2011, 2012, 2013 and 2014, respectively. Of these poisonings, 9, 8, 1 and 1 cases were attributed to OR intoxication in 2010, 2011, 2012 and 2013, respectively (Bronstein et al., 2012; Bronstein et al., 2011; Mowry et al., 2013; Mowry et al., 2014). From the above information it is clear that OR poisoning has emerged as a public health problem worldwide.

1.2 *Cortinarius* species

OR is a highly nephrotoxic toxin found in various mushrooms of the *Cortinaceae* family, genus *Cortinarius* and subgenus *Leprocybe* (Karlson-Stiber and Persson, 2003), including fool´s webcap (*Cortinarius orellanus*) and deadly webcap (*Cortinarius rubellus* formerly named *Cortinarius speciosissimus* or *Cortinarius orellanoides*) (Barceloux, 2008; Herrmann et al., 2012).

Cortinarius species is the largest genus of fungi that forms mushrooms comprising over 250 species. These usually form mycorrhizae with trees and can be found in Northern Europe and in mountainous areas of Central Europe (Barceloux, 2008), as well as in Northern America at the end summer or autumn (Horn et al., 1997). *Cortinarius rubellus* species inhabit moist or west conifer forest, particularly in moist acidic soil (Barceloux, 2008).

The genus *Cortinarius* has a variety of shapes, but the common features are a spider-web lie veil, the "*cortina*", connecting the margin of the cap to the stalk, particularly in young specimens (Figure 1(a)). As the mushroom ages, the *cortina* disappears, resulting

in almost indistinguishable remains of the *Cortina (*Figure 1(b)) (Barceloux, 2008). The genus is subdivided into a number of subgenera using cap and stipe features of dry or viscid and base of the stipe bulbous or non-bulbous. Added to these are pigmentary differences, microscopic cap features and differences in ultraviolet fluorescence (Duffy, 2008).

They also have an orange, purple, or greenish yellow-colored fruit bodies with characteristic rusty orange gills and a thick stalk with striae and bulbous base (Wornle et al., 2004). These are characterized by having a knob-like protuberance on the rust-to yellow-brown cap (2-12 cm/1-5 in) that is conical in shape on young species and more flattened on older specimens. The broad, elliptical spores yield a rust brown spore print. The cylindrical stalk is similar in color to the cap and stalk sometimes contains lemon yellow bands that represent the remains of *Cortina* (Barceloux, 2008).

OR has been found to be the toxin responsible for the lethal toxicity of several species of *Cortinarius* mushrooms. Only some species of *Cortinarius* mushrooms, such as *Cortinarius orellanus*, *C. rubellus* (*C. orellanoides, C. speciosissimus, C. rainierensis, C. henrici*), *C. brunneofulvus*, *C. splendens*, *C. cinnamomeus, C. atrovirens*, *C. venenous*, *C. gentilis* and *C. armillatus* (Antkowiak and Gessner, 1985; Bresinky and Besl, 1990; Danel et al., 2001; Oubrahim et al., 1997; Shao et al., 2016) contain the nephrotoxin OR.

The content of OR was determined in dried fungus, varying greatly depending both on the mushroom portion considered and *Cortinarius* species. The yield of OR in these species is usually reported as being approximately 2% of the dried mushroom; the content is slightly lower in *C. speciosissimus* (Prast et al., 1988). The analysis of *C. orellanus* and *C. rubellus* species showed that the content of toxin (expressed on dry weight basis) was 9400 and 7800 mg/Kg in caps, 4,800 and 4,200 mg/Kg in stems, and 3100 and 900 mg/Kg in spores, respectively. In mycorrhiza roots from *C. rubellus*, the OR content was 300 mg/Kg (Koller et al., 2002). In another study, the OR content in dried fungus was found to be about 14000 mg/Kg in *C. orellanus* and 9000 mg/Kg in *C. rubellus* (Prast and Pfaller, 1988).

1.2.1 *Cortinarius orellanus*

C. orellanus (Figure 1 (c)) is a medium sized gill mushroom that is characterized by a domeshaped, dry, orange, red, brown-to-yellow, brown cap; thick, well-spaced, orange brown gills, and a dry, yellowish to reddish brown stalk that is tapered below with some veil fibrils on the surface. The body of the mushroom is whitish to yellowish. Species in

this group have rust brown spore prints and the spores are elliptic and distinctly ornamented (Judge et al., 2010). The cap is 3-8 cm across and stalk measures 3-9 cm long and 4-12mm thick. These mushrooms fluoresce blue ultraviolet light (Duffy, 2008).

C. orellanus is rare in Scandinavia but more common on the continent of Europe. It grows preferably in deciduous forest (Holmdahl, 2001).

1.2.2 *Cortinarius speciossimus* or *rubellus*

C. speciossimus (Figure 1 (d)) is characterized by a cap rust brown to orange, often has a steeper and darer colored elevation at the top of the cap, and its surface is dry and slightly scaly. The cap diameter is typically 4-8 cm when fully expanded, and the margin is often slightly rolled down even in fully mature specimens. The gills are pale yellowish at first, becoming rusty brown as the spores mature (Philips, 2006). It has been claimed that both *C.henrici* (Figure 1 (e)) and *C. rainierensis* are identical to *C. rubellus* (Karlson-Stiber and Persson, 2003).

C. speciosissimus is a boreal fungus of coniferous forests, being widely distributed in the northern areas of central Europe (*i.e.* Scandinavia, northern Scotland) and in the mountainous areas of central Europe. Its prevalence seems to have increased in the most recent decades in Sweden, specially on acid soils in the southwestern parts (Holmdahl, 2001).

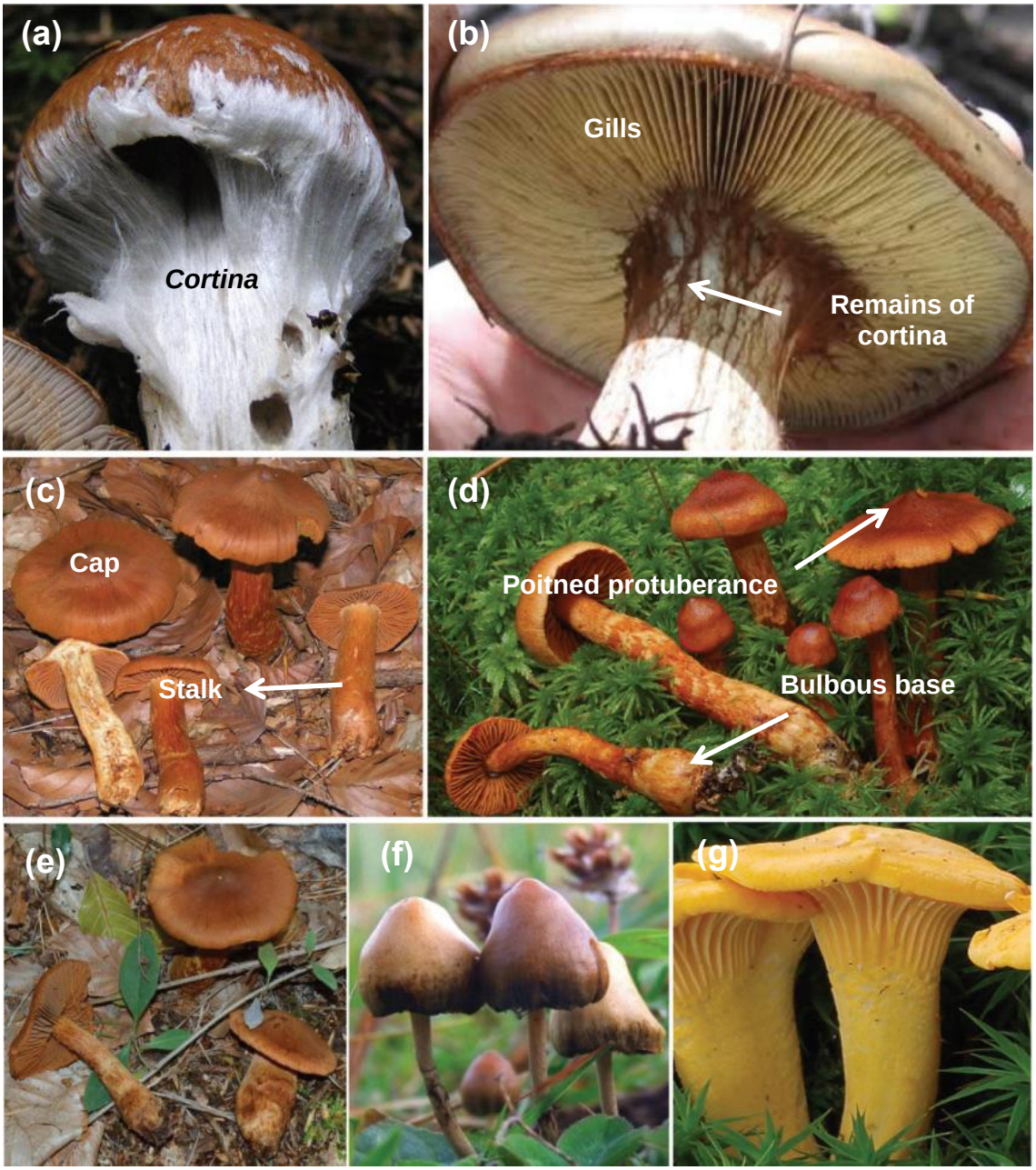


Figure 1. (a) *Cortina*; (b) Remains of *cortina*; (c) *Cortinarius orellanus*; (d) *Cortinarius rubellus;* (e) *Cortinarius henrici;* (f) *Psilocybe semilanceata*; and (g) *Cantharellus cibarius*.

1.3 Orellanine

1.3.1 Chemistry

Table 1 presents chemical properties of OR. It is a bipyridine N-oxide (2,2'-bipyridine-3,3',4,4'-tetrahydroxy-1,1'-dioxide) that exists as two tautomers (Nilsson et al., 2008) (Figure 2). The more stable tautomer is the amine oxide form. OR is a colorless fine

crystalline and navy blue-fluorescing compound that is stable at 150-160ºC, decomposing slowly above this temperature and under UV light to the yellow nontoxic bipyridyl compound orelline by releasing oxygen. An explosive degradation occurs at temperatures over 267ºC (Antkowiak and Gessner, 1985). Cooking temperatures, freezing and drying do not reduce the orellanine content (Richard et al., 1988). OR is stable for many years in the intact mushroom (Schumacher and Hoiland, 1983). Figure 3 demonstrates the reduction of OR to the nontoxic compound, orelline, via the intermediate orellinine (Antkowiak and Gessner, 1985; Oubrahim et al., 1998; Rapior et al., 1989; Richard et al., 1988). Orelline appears as bright yellow crystals and orellinine is almost colorless. The stereochemistry of OR was confirmed by X-ray crystallography (Cohen-Addad et al., 1987). In crystal conformation, the planes of the two pyridyl rings are nearly perpendicular, making the molecule chiral. However, OR isolated from the mushroom is an optically inactive racemic mixture; this is probably due to the low rotational barrier to racemization (Konig et al., 1994).

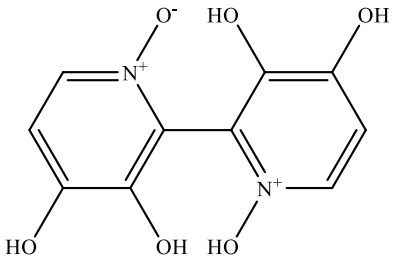
Table 1 – Chemical properties of orellanine.

Molecular formula	$C_{10}H_8N_2O_6$
Molar mass	252.2 g/mol
CAS number	37338-80-00
Density	1.777g/cm^3
Boiling Point	834.6ºC at 760 mmHg
Flash Point	458.6ºC
Enthalpy of Vaporization	125.5 kJ/mol
Polar Surface Area	131.8 Å^2
Index of Refraction	1.7
Molar Refractivity	56.6 cm^3
Molar Volume	141.1 cm^3
Polarizability	22.4 ×10^{-24}cm^3
Surface Tension	86.8 dyne/cm
log P	- 0.53
log P	- 1.2
pKa (Strongest Acidic)	- 9.3
pKa (Strongest Basic)	- 3

In 1962, Grzymala (Grzymala, 1962) was the first to demonstrate experimentally the nephrotoxicity of *C. orellanus* and was the first to isolate OR on biological analysis. Dehmlow and Schulz (1985) were the first reporting the successful synthesis of OR and orelline (Dehmlow and Schulz, 1985). OR was synthesized in nine steps from 3-amino pyridine. Orelline has also been synthesized from 2-bromo-3-hydroxypyridine (Hasseberg and Gerlach, 1988).

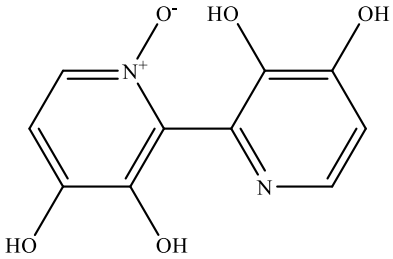
As referred, when placed under UV light, OR is, at first, navy blue fluorescent, and after several minutes shifts to bright turquoise fluorescent due to a rapid photochemical reaction (Antkowiak and Gessner, 1975). This photochemical decomposition has been shown to proceed by stepwise loss of the N-oxides to give, first, the mono N-oxide orellinine and, subsequently, orelline (Antkowiak and Gessner, 1985).

OR, orellinine and orelline are soluble in dilute sodium hydroxide, ammonium hydroxide and dimethyl sulfoxide (DMSO), slightly soluble in methanol, and practically insoluble in most organic solvents and water (Antkowiak and Gessner, 1979; Schumacher and Hoiland, 1983). OR, contrary to orelline, is well soluble in alkaline solutions.



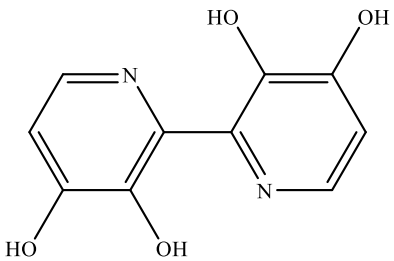
Orellanine

reduction



Orellinine

reduction



Orelline

Figure 2. Chemical structures of orellanine, orellinine and orelline.

1.3.2 Toxicokinetics

Little is known about the toxicokinetics of OR in humans, specially during the first 24 hours after ingestion (Ojanperã, 2008; Prast and Pfaller, 1988). In animal studies, kidneys excrete detectable quantities of OR only for the first 24 hours after exposure (Prast and Pfaller, 1988). This is consistent with observations in human cases of OR poisoning, where the toxin is not detected in urine samples collected from 2 to 18 days after ingestion (Dickman and Grollman, 2010; Rohrmoser et al., 1997). Typically, clinical samples are not obtained until several days after ingestion, due to the delayed onset of symptoms. Indeed, this has led to misdiagnosis of the poisoning, since the victim often does not associate the symptoms with a meal taken several days before. For this reason, no information is available regarding human plasma levels of OR attained shortly after exposure (Dickman and Grollman, 2010).

Based on ingestion of a toxic dose of 100-200 g of mushrooms containing 0.1% OR, and assuming complete absorption and full plasma distribution, maximal peak plasma levels of the toxin is expected to range from 130 to 260 µmol/L (Grzymala, 1957; Koller et al., 2002). Based on limited testing, the clearance of OR from the blood is probably rapid (*i.e.*, < 2-3 days) (Karlson-Stiber and Persson, 2003).

The continued presence of OR in renal cortex after hemodialysis, and its persistent presence in this tissue for several months in the absence of detectable OR in urine or blood, suggests that the toxin is sequestered in the kidney in a poorly exchangeable form (Dickman and Grollman, 2010).

1.3.3 Toxic doses

Table 2 resumes the toxic doses of OR and dried *Cortinarius* homogenates for different species. The LD_{50} has been determined in different animals and by different routes. Several studies on the toxicity of *C. rubellus* in rats were carried out by Nieminen and collaborators (Mottonen et al., 1975; Nieminen, 1976; Nieminen and Pyy, 1976a). Authors have shown that rats appear to be resistant to *Cortinarius* poisoning and a genetic variability was suggested since females were more resistant (Nieminen et al., 1976). In another study (Nieminen and Pyy, 1976b), 23% of the animals showed total resistance to toxins, irrespective of dose, whereas in others the observed renal lesions were dose-dependent.

Table 2. Toxicity of orellanine and dried *Cortinarius* homogenates. Adapted from the work by Lampe *et al. (Lampe, 1991).*

Toxin	Species	Route	LD_{50}	LD_{100}	Remarks
OR	Mouse	SC	8.3 mg/Kg		
		PO	33 mg/Kg	109 mg/Kg	Male and female mice
		PO	90 mg/Kg		
		IP	15 mg/Kg	42 mg/kg	Male and female mice
		IP	12.5 mg/Kg		
	Cat	PO	8.3 mg/Kg		
	Guinea pig	IP	8 mg/Kg		
C.orellanus	Mouse	PO	2.2 g/Kg	4.57 g/Kg	Containing 14 mg/g orellanine; male mice Containing 15-20 mg/Kg orellanine; female mice Containing 15-20mg/g orellanine; male rats
		PO	2.1 g/Kg		
	Rat	PO	0.976 g/Kg		
C.rubellus	Mouse	PO	3.12 g/Kg	6.22 g/Kg	Containing 9 mg/g orellanine; male mice

SC: subcutaneo; PO: *per or*;IP: intraperitoneal; LD_{50}: medianl ethal dose; LD_{100}:absolute lethal dose

Clinical data indicates that humans appear to be more sensitive to toxic effects than mice and rats. The ingestion of only two to three mushrooms (3 mg of toxin which corresponds to 0.04 mg/kg for a human of 70 kg) seems to be enough to make the victim dependent on dialysis for the entire life (Calvino et al., 1998; Delpech et al., 1990). Similarly, in humans, the greater susceptibility of males to *Cortinarius* toxicity has been described by several authors (Danel et al., 2001; Short et al., 1980). Herrmann and colleagues (Herrmann et al., 2012) described a lethal dose of fresh *C. rubellus* between 29 and 227 g for humans weighting about 70 kg.

1.3.4 Mechanism of toxicity

OR and orellinine have similar toxicity, but orelline proved to be nontoxic (Spoerke, 2000). The mechanism of toxicity is not yet fully understood. It was shown that the toxin inhibits the synthesis of macromolecules such as proteins, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) (Danel et al., 2001; Karlson-Stiber and Persson, 2003; Richard et al., 1991); promotes a noncompetitive inhibition of the activity of alkaline phosphatase, γ-glutamyl transpeptidase and leucine aminopeptidase (Ruedl et al., 1989); and interrupts the production of adenosine triphosphatase (Spoerke and Rumack, 1994), namely at the proximal tubular brush border, compromising utilization and reabsorption of peptides, polysaccharides and other molecules.

Moser (Moser, 1969) showed that *C. orellanus* toxin inhibits DNA-depended activity of RNA polymerase B from rat liver (eukaryotic cell) and DNA-depended activity of RNA polymerase from *Escherichia coli* (prokaryotic cell). Later, Richard and co-authors (Richard et al., 1991) pretreated rat liver microsomes with OR and then exposed rabbit reticulocyte lysate to this mixture. It was observed the inhibition of protein synthesis, while direct addition of untreated OR or only microsomes did not. This suggests that inhibition of protein synthesis is due to a metabolite of OR.

Orellanine inhibits pinocytosis in *Amoeba proteus* and inhibits growing of both slime mold *Discyostelium discoideu* and *Escherichia coli* at 80 μM (Spoerke and Rumack, 1994). The large spectrum of toxic effects in plants, animals and microorganisms suggests that the target is likely to be a cellular process found in both prokaryotes and eukaryotes (Richard et al., 1988).

Rapidor and co-authors (Rapior et al., 1988) suggested that the *Cortinarius* toxicity is caused by metabolites with the isoxazolium core derived from the photochemical rearrangement of orellanine (called the phototoxicity mechanism of OR). In accordance, authors found that OR purified in the dark and administered to laboratory animals show low toxicity, while the one extracted in the light induces a toxic response (Figure 3). These intermediates can bind covalently with numerous proteins in the body, leading to organ damage.

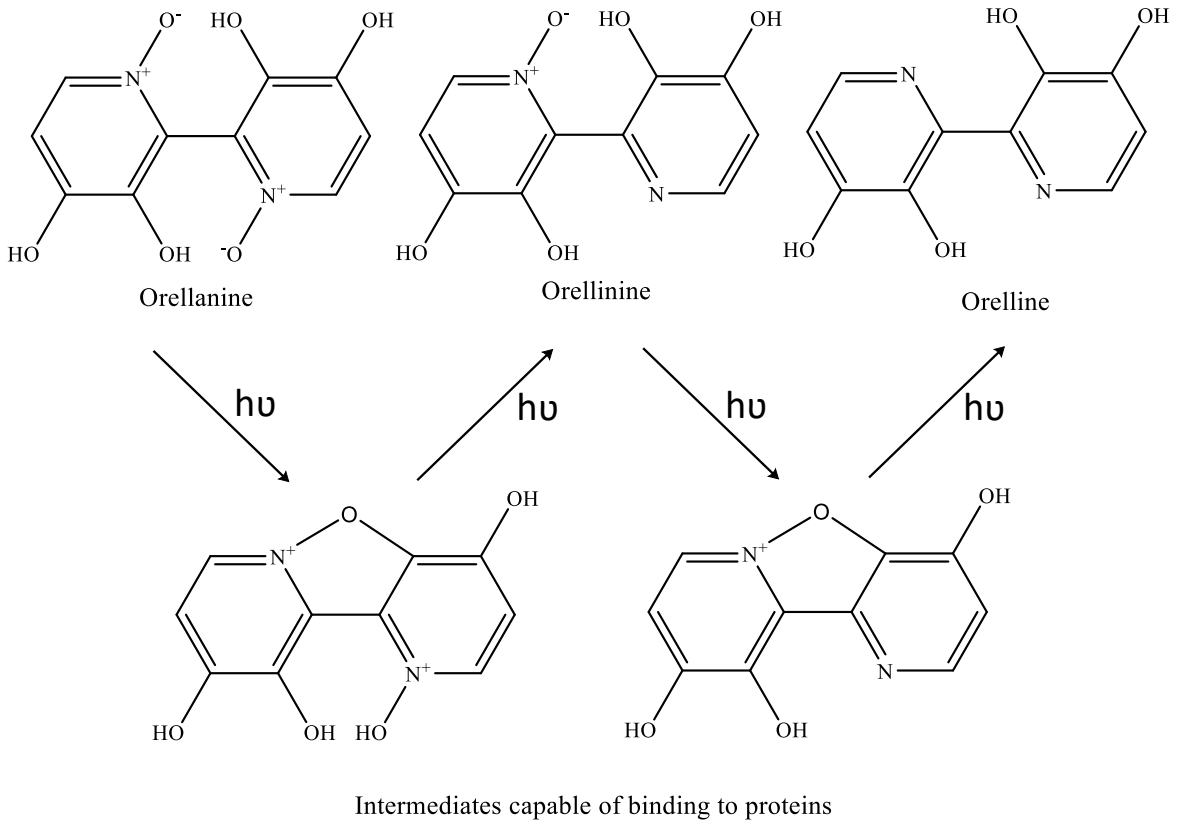
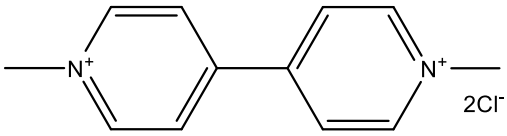
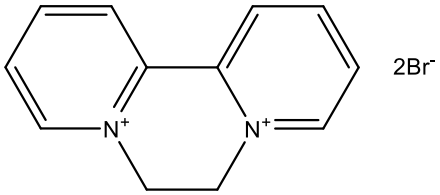


Figure 3. Phototoxicity mechanism of orellanine.

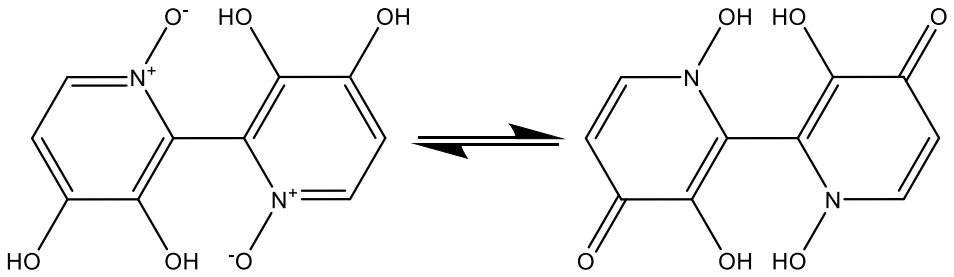
As already referred, OR chemically resembles the pyridine herbicides diquat (1,1'-ethylene- 2,2'-bipyridinium) and PQ (1,1'-dimethyl-4,4'-bipyridinium) (Figure 4), and some authors suggested a possible mono-electronic reduction mechanism (generation of a stable radical) for its toxicity mechanism (Richard et al., 1995; Schumacher and Hoiland, 1983). The consequence is obvious: production of free radicals and, therefore, oxidative stress similarly to PQ (Dinis-Oliveira et al., 2008; Dinis-Oliveira et al., 2009b). In opposite to PQ, this proposed intracellular toxic mechanism with NADPH plus H^+ depletion would need a long time to impoverish the cells to the degree of inevitable necrosis, according with the delayed toxic effects observed in this type of mushroom poisoning (Deshpande, 2002).



Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride)



Diquat (11,1'-ethylene-2,2'-bipyridylium dibromide)



Orellanine (3,3',4,4'-tetrahydroxy-2,2'-bipyridine-N,N'-dioxide)

Figure 4. Chemical structures of paraquat, diquat and orellanine.

However, this model has been criticized since OR has a much more negative redox potential than PQ and diquat (Cantin et al., 1988; Richard et al., 1997). Noteworthy, it was observed the formation of a radical form of OR by near UV (370 nm) at physiological pH under aerobic or anaerobic conditions (Richard et al., 1997). This apparently stable radical, identified as *ortho*-semiquinone anion radical (Figure 5), was also generated using biological oxidizing agents (*e.g.*, cytochrome c and nicotinamide adenine dinucleotide) or enzymatic systems such as tyrosinase/oxygen (O_2) and peroxidase/hydrogen peroxide (H_2O_2) (Oubrahim et al., 1998; Richard et al., 1995). Indeed, it is the *ortho*-semiquinone anion radical the responsible for superoxide radical production. At the renal spot of bioaccumulation of the toxin, a quickly change of small portions of OR rapidly cycling from its oxidized to its reduced form, might be sufficient to support continuous production of oxygen free radicals, which will induce oxidative stress. This process may lead to a large oxygen consumption, which might create hypoxic conditions, as well as the dramatic depletion of renal glutathione and ascorbate levels, consequently making cells more

susceptible to oxidant damage (Cantin et al., 1988; Oubrahim et al., 1998; Richard et al., 1995). Corroborating this hypothesis, OR-treated animals did experience increased oxidative stress which is indicated by increasing of both plasma levels of ascorbyl radicals and protein oxidation in renal tissue (Nilsson et al., 2008).

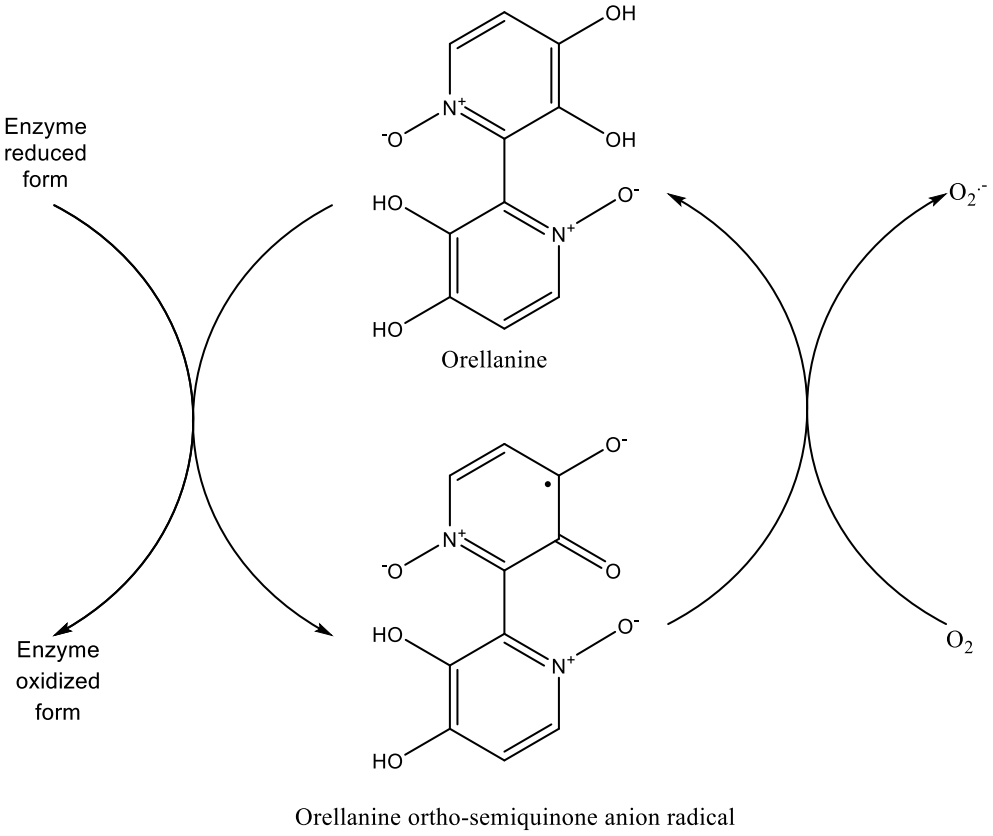


Figure 5. Redox cycling of orellanine.

Oubrahim and colleagues (1997) proved the formation of a characteristic *ortho*-semiquinone radical anion ($SQ^{\cdot}$) derived from OR in a system containing horseradish peroxidase and H_2O_2. Since peroxidative oxidation proceeds *via* two one-electron oxidation steps, this enzyme can oxidize two molecules of OR to the corresponding semiquinone during one enzymatic cycle, or one molecule of orellanine to the corresponding quinone by two successive monoelectronic oxidation steps. The *ortho*-semiquinone form of OR was also generated by the enzymatic system tyrosinase/O_2 which proceeds via a two-electron oxidation. In this case, the generation of the *ortho*-semiquinone radical likely occurs as a result of an equilibrium between OR and its corresponding quinone. During oxidation of OR in renal tissue, *ortho*-semiquinone likely accumulates, whatever the mode of enzymatic oxidation may be (one or two electrons). The *ortho*-semiquinone, as well as the quinone, could participate in a variety of reactions

including covalent binding to biological compounds, leading to cell damage. In addition, when oxidation of orellanine by horseradish peroxidase/H_2O_2 occurred in presence of reducing agents of biological interest, such as ascorbic acid or glutathione, the formation of ascorbyl or glutathionyl radicals was observed (Oubrahim et al., 1997).

Although all authors agree that OR is the main toxin, *Cortinarius* species also contain cyclic decapeptides (cortinarin A, B and C) that produce renal damage (Caddy et al., 1982; Tebbett and Caddy, 1984). Cortinarin A and B (not C) were found to be nephrotoxic in animal studies (Tebbett and Caddy, 1984), but the role of these decapeptides in causing the *Orellanus* syndrome, or even their existence, have been questioned by several authors (Matthies and Laatsch, 1991).

1.4 Poisoning orellanine

1.4.1 Epidemiological studies

OR poisoning is extremely serious. Until today orellanine poisoning has occurred in most parts of the European continent, Britain, North America, Canada and Australia (Bednarova et al., 1999; Danel et al., 2001; Esposito et al., 2015; Moore et al., 1991; Mount et al., 2002; Raff et al., 1992).

Intoxication with the fungus C. *orellanus* has been described in literature. The toxicity of the mushroom C. *orellanus* was firstly discovered in 1957 when 136 habitants of Bygdosz (Poland) were intoxicated, which resulted in 23 deaths (Grzymala, 1957; 1959b; 1962; Grzymala, 1965a; Grzymala, 1965b; Wysocki et al., 1958). *C. orellanus* intoxications are reported from Switzerland (Favre et al., 1976), France (Andary et al., 1989; Bouget et al., 1990; Brousse et al., 1981; Delpech et al., 1990; Marichal et al., 1977; Rapior et al., 1989), Germany (Eigler et al., 1997; Farber and Feldmeier, 1977), Czechoslovakia (Bednarova et al., 1999; Bouska et al., 1979; Stredova et al., 1978), Bulgaria, Austria (Franz et al., 1996; Horn et al., 1997; Kerschbaum et al., 2012; Rohrmoser et al., 1997), Spain (Calvino et al., 1998), England (Kilner et al., 1999), Italy (Montoli et al., 1999) and U.S.A. (Moore et al., 1991).

In 1974, four cases of poisoning by *C. rubellus* occurred in Finland (Hulmi et al., 1974), followed by five cases in 1992 (Tidman and Sjostrom, 1992). Further incidents were reported in Scotland (Short et al., 1980; Watling, 1982), Sweden (Holmdahl and Blohme, 1995; Holmdahl et al., 1984), Norway (Fauchald and Westlie, 1982; Jacobsen et al., 1981), Italy (Busnach et al., 1983; C. et al., 1984), Germany (Nolte et al., 1987),

France (Traverso, 1973) and United Kingdom (Nagaraja et al., 2012), Scotland (Short et al., 1980) and Austria (Franz et al., 1996; Holzl et al., 1997; Kerschbaum et al., 2012).

Intoxications by a further species, *C. splendens*, has been described in France (A., 1981; Colon et al., 1981; 1982) and Switzerland (Schliessbach et al., 1983).

Nephrotoxic *Cortinarius* species are morphological similar to mushrooms from *psilocybe* genus (*Psilocybe semilanceata*) (Figure 1 (f)), leading to mistake by inexperienced hallucinogenic mushrooms hunters (Franz et al., 1996). Likewise, misidentification of *C. orellanus* as *Cantharellus cibarius* (Figure 1(g)) and *Cantharellus tubaeformis* also occurs (Barceloux, 2008; Horn et al., 1997). It can be concluded that cases of orellanine poisoning are due to a lack of knowledge of mushroom species. This fact has led to several cases of accidental intoxication (Franz et al., 1996).

1.4.2 Prognosis

The chance of full recovery from OR poisoning depends on the amount of ingested toxin, age and general health of the individual patient, probably preexisting subclinical renal disease, and the time at which therapy is started (Horn et al., 1997).

Grzymala (1959 and 1965) established a relationship between the duration of the latent period and poisoning severity (Grzymala, 1959a) (Gryzmala, 1965; Grzymala, 1965b) . In his series, the latent period was:

a) 10-17 days in patients presenting with thirst, burning sensation in the mouth and polyuria (mild intoxication);
b) 6-10 days in patients presenting with digestive disorders, polyuria or oliguria, hematuria and leucocyturia, but no significant renal impairment;
c) 2-3 days in patients with acute renal failure and death rate of about 50%.

A short latent period indicates more severe poisoning. However, the rather variable individual response to orellanine poisoning makes an assessment of outcome difficult.

Draffan and colleagues (Draffan et al., 1977) developed a method to measure bipyridines in plasma, being possible to get an exact measurement of the OR content in plasma. This method can give a good indication of the severity of poisoning, and also be a guide for a better treatment (Schumacher and Hoiland, 1983).

Holdmdahl (2001) proposed a method for estimating the prognosis in individual cases. This method is called the "*Cortinarius* nephron toxicity prognostic index (CNT)" and is based on two parameters general available. The parameters used are the serum creatinine level before treatment and the former days after ingestion of the mushrooms. It

is calculated from the formula CNT=$(y + 316)/X \times 10^4$), where y is the serum creatinine and X the number of elapsed days. CNT index < 1.1 indicates a good prognosis; CNT between 1.1 and 2.1 indicates "intermediate" prognosis (probably chronic renal failure with serum creatinine higher than 200 µmol/L) and > 2.1 a poor outcome with end-stage renal failure requiring renal replacement therapy (Holmdahl, 2001).

1.4.3 Diagnosis

The diagnosis of OR poisoning can be clinical, mycological or toxicological (Herrmann et al., 2012; Rapior et al., 1989; Wornle et al., 2004).

1.4.3.1 Clinical diagnosis

OR poisoning is characterized by a long latent period. Typically, the onset of symptoms is delayed from 2-4 to 14 days after ingestion (Danel et al., 2001). The higher the quantity of mushrooms consumed, the shorter the latent period (Lampe, 1991). There is a significant variation on individual responses to poisoning, with the degree of renal injury ranging from mild and transient to severe and irreversible (Dickman and Grollman, 2010).

Some patients suffer mild gastrointestinal discomfort in the latency period before developing signs of renal impairment. Table 3 resumes signs, symptoms and analytical findings related to OR poisoning. The gastrointestinal phase (also known as prerenal phase) is primarily characterized by vomiting and nausea, and, less often, abdominal pain, asthenia, chills, burning sensation in the mouth, thirst, headache, myalgia, anorexia and diarrhea (Danel et al., 2001; Holmdahl and Blohme, 1995; Karlson-Stiber and Persson, 2003; Schumacher and Hoiland, 1983).

Table 3 – Signs, symptoms and analytical findings in intoxications by orellanine.

Prerenal phase
Vomiting, polydipsia, lumbar pain, nausea, abdominal pain, headache, polyuria, asthenia, diarrhea, anorexia, myalgia, faintness, paresthesia, constipation, chills, somnolence, vertigo, dysgeusia, sweats, tinnitus, burning in mouth, fatigue, thirst, dry mouth, visual defects, lumbar pain
Renal phase
Myalgia, intense lumbar pain, flank pain, oliguria, leukocyturia, hematuria, proteinuria, anuria, glycosuria, leukocytosis, increased serum creatinine, potassium and urea, renal histopathological analysis evidencing tubule-interstitial nephritis, interstitial

edema, inflammatory infiltrates and fibrosis/sclerosis
Treatment
Hemodialysis/peritoneal dialysis, extracorporeal hemoperfusion, plasmapheresis, corticosteroids, diltiazem, dopamine, selenium N-acetylcysteine and renal transplant

Clinical signs may spontaneously disappear in some patients, leaving the poisoning unnoticed; in others, the signs become more intense and are accompanied by neurological manifestations (*e.g.*, paresthesia, taste impairment, cognitive disorders and dizziness), lumbar pain and anuria requiring hospitalization (Danel et al., 2001; Holmdahl and Blohme, 1995; Michelot and Tebbett, 1990).

Renal failure may develop several days to weeks after initial symptoms due to OR sequestration in the kidney. The incidence of renal failure varies from 30-70% of patients, and 30-65% need temporary dialysis (Danel et al., 2001). Renal involvement is the other peculiarity of this poisoning and is mainly characterized by lumbar and flank pains, intense thirst, oliguria, polyuria, proteinuria, hematuria and leukocyturia (Carter et al., 1983; Danel et al., 2001; Judge et al., 2010; Michelot and Tebbett, 1990; Schumacher and Hoiland, 1983). The blood balance shows an increase in urea and creatinine levels (Judge et al., 2010; Michelot and Tebbett, 1990). Differential diagnosis with other nephrotoxic compounds, such as oxalates crystals found in certain plants (e.g. genus *Philodendron* and *Dieffenbachia*) and ethylenoglycol found in antifreeze products, is relatively easy. In these poisonings, signs and symptoms are evident during the first hours after exposure and circumstantial evidences can help diagnosis (Nelson and Goldfrank, 2011).

Nephrotoxicity is characterized by an early and severe interstitial fibrosis, interstitial edema and tubular epithelial necrosis (Carter et al., 1983; Michelot and Tebbett, 1990; Rapior et al., 1989; Schumacher and Hoiland, 1983). Short and colleagues (1980) have shown that, when initial biopsy was made until 2-3 weeks after poisonings, specimens exhibited pronounced focal tubular damage with tubulorrhexis, cast formation and severe interstitial edema with patchy infiltration of lymphocytes, plasma cells and some polymorphs (Short et al., 1980) . The glomeruli showed only slight mesangial cell reaction. In the later biopsy specimens (obtained 7-8 weeks after poisoning), the mild glomerular reaction was still present but the major features were tubular dilatation and cellular atrophy with groups of apparently normal tubules between damaged areas. In both cases the interstitial edema was much less in the later specimens, but there was early fibrosis between the damaged tubules. A mild mononuclear inflammatory cell infiltration was present. Immunofluorescence studies showed no significant deposition of immunoglobulin, complement or fibrin in glomeruli, but immunoglobulin G, immunoglobulin

A and fibrin were isolated from tubular casts (Short et al., 1980). Therefore, the existence of renal alterations as a consequence of some immune reaction cannot be excluded.

In poisoning due to *C. rubellus*, most cases have not been admitted to hospital before 8-14 days after the mushroom ingestion and then the clinical picture is that of an acute renal failure (Schumacher and Hoiland, 1983). It is estimated that 30-45% of individuals who ingest nephrotoxic *Cortinarius* mushrooms develop acute renal failure (Danel et al., 2001). Of these, half usually recover the renal function and half progress to chronic renal insufficiency and require maintenance hemodialysis or kidney transplant (Dickman and Grollman, 2010).

Some patients may become asymptomatic and the renal injury is only identified by biochemical tests (Nagaraja et al., 2012).

In a study of 26 patients with nephrotoxicity secondary to *Cortinarius* mushroom ingestion, the incidence of end-stage renal failure requiring dialysis and renal transplantation was approximately 8 (Bouget et al., 1990), whereas 9 of 22 Swedish patients (41%) developed end-stage renal disease after ingesting mushrooms from *Cortinarius species* (Holmdahl and Blohme, 1995).

Liver injury has also been observed based on increase of transaminases and bilirubin levels, hepatomegaly, hepatalgia, and lipoidosis and necrosis lesions evidenced by histological analysis (Gryzmala, 1965; Grzymala, 1965b), but most of studies ruled out liver involvement (Danel et al., 2001).

Worth mentioning, OR is currently being tested as a potential treatment for metastatic renal cancer based on its highly selective toxicity to renal cells (Hedman et al., 2012).

1.4.3.2 Mycological and toxicological analysis

Several analytical methods were developed for the analysis of both mushrooms and biological samples, such as serum, renal tissue and stomach contents. The ferric-OR reaction could be useful for both mycologists and medical personnel by demonstrating whether an unknown *Cortinarius* specimen contains, or not, OR. Schumacher and Hoiland (1983) have proposed a rapid qualitative test to detect OR in mushrooms. A fresh or dried mushroom is crushed in 5 volumes of water and filtered after 10 minutes at room temperature. The filtrate is then mixed with an equal amount of 3% ferric chloride hexahydrate ($FeCL_3$:$6H_2O$) dissolved in 0.5N hydrochloric acid (HCl) (Schumacher and Hoiland, 1983). The presence of OR is suspected if a dark grey-blue color ink appears. However, other authors described an immediate change of the yellowish liquid to purple-red or violet (Kirchmair and Poder, 2011).

Thin-layer chromatography can be used for separation prior to proof of the presence of toxins under UV light. A simplified procedure for detecting OR is as follows: a small piece of dried fungus is crushed and extracted in 50% ethanol, allowed to stand for 15 min at room temperature, and then the extract is applied to a silica gel plate and chromatographically developed by n-butanol:acetic acid:water (3:1:1). After drying, the plate is sprayed with 2% $FeCl_3$ in 0.5 N HCI. OR is visible as a navy blue spot from the application point (retention factor (R_f) of 0.25-0.5), orellinine as a dark blue spot, and orelline as a light blue spot (Horn et al., 1997; Schumacher and Hoiland, 1983).

Other analytical procedures are available, such as electrophoresis (Oubrahim et al., 1997) and high-performance liquid chromatography (HPLC) with photodiode array (Koller et al., 2002), electrochemical (Holmdahl et al., 1987) and UV (Cantin et al., 1989) detection. In these last two methods, reversed-phase ion-pair HPLC with phosphate-containing eluent20 and amide, and C18 columns with phosphoric acid as the eluent10, were employed. OR has also been detected in single MS mode using electron impact (Holmdahl et al., 1987) and electrospray ionization (Herrmann et al., 2012; Horn et al., 1997; Koller et al., 2002).

Brondz *et al* developed two methods, HPLC-MS and GC-MS, to analyze rat stomach content samples, after ingestion of *C. orellanus* (Brondz and Brondz, 2012; Brondz et al., 2012).

In a renal biopsy specimen, OR can be detected up to 6 months after poisoning by performing a thin-layer chromatography technique (Andary et al., 1989; Rapior et al., 1989; Rohrmoser et al., 1997). Orelline, the di-reduction metabolite, has also been found in renal biopsies in cases of OR poisoning, and possibly originates from either the mushroom itself or from extra- or intrarenal metabolism of OR (Dickman and Grollman, 2010).

Since OR is rapidly concentrated in the kidney, it will not be detected neither in urine nor in blood and dialysis fluids at the time when the first symptoms appear (Frank et al., 2009). Toxin in plasma can be only detected for up to 2 weeks after ingestion (Andary et al., 1989; Rapior et al., 1989). However, Andary and colleagues (1989) were able to detect a plasma OR concentration of 20 µmol/L in a sample obtained from a patient 9 days after eating two mushrooms, and they used hemodialysis to effectively clear the circulating toxin. The toxin is not detected in urine samples collected as early as 2 days after ingestion and as late as 18 days (Rapior et al., 1989).

Light microscopy has also been useful to highlight characteristic renal histopathological findings, such as acute renal injury and interstitial edema and invasions of inflammatory cells with interstitial nephritis (Frank et al., 2009).

1.4.4 Treatment

There is no specific antidote to OR poisoning, treatment being mainly supportive care as well as the use of hemodialysis, as needed. Prolonged monitoring of renal function is necessary because of the slow resolution of kidney dysfunction (Barceloux, 2008).

Emesis or gastric lavage might, in theory, be indicated if the patient is seen earlier than 6 hours after ingestion. Oral activated charcoal may be used if given early, although its efficacy has not been established (Spoerke and Rumack, 1994).

Extracorporeal hemoperfusion, hemodialysis and plasmapheresis are techniques used to remove the toxin from circulation but should only be considered if the patient is seen within 1 week after ingestion (Busnach et al., 1983; Fulde et al., 1998; Horn et al., 1997; Kilner et al., 1999; Montoli et al., 1999; Rapior et al., 1989). Beyond that period, the use of hemodialysis depends only on the need to support renal function (Barceloux, 2008). About half of the patients requiring dialysis did not recover kidney function (Danel et al., 2001). Heath and colleagues reported the successful treatment of two persons by hemoperfusion over resin filters started up 5 days after ingestion of *C. speciosissimus* (Heath et al., 1980). In the series of *C. orellanus* poisonings reported by Bouget *et al*., of the 12 patients who had acute renal failure, 8 required hemodialysis (Bouget et al., 1990). Rapidor *et al.* reported a case of acute renal failure after ingestion of two *C. orellanus* mushrooms. The patient was treated on day 10 by hemodialysis and charcoal hemoperfusion. The OR serum level was 6.12 mg/mL before hemodialysis, and no toxin could be detected after treatment (Rapior et al., 1989).

There are also reports of cases where the use of corticosteroids, N-acetylcysteine and selenium allowed clinical improvement (Kilner et al., 1999; Wornle et al., 2004). N-acetylcysteine is a glutathione donor and antioxidant (Richard et al., 1995) and the selenium is an essential component of several major metabolic pathways including immune and antioxidant defense systems (Brown and Arthur, 2001). It has been shown that selenium improves the clinical outcome of patients with severe inflammatory response syndrome (Angstwurm et al., 1999). Wornle *et al.* demonstrated that therapy with selenium contribute to the subsequent improvement in renal function of patients intoxicated with *C. specisossimus* (Wornle et al., 2004). In the series reported by Bouget and colleagues, a treatment with corticosteroids (prednisolone, 10mg/Kg/day for 3 days and 1 mg/Kg/day for 3 weeks) initiated in nine patients between the 11^{th} and 19^{th} day post ingestion, did not change the evolution of the renal failure (Bouget et al., 1990). N-

acetylcysteine was given in one case on the 11th day without any better efficacy (Kilner et al., 1999).

It has been emphasized that forced diuresis could rather accelerate and amplify the nephrotoxic process, since it possibly accentuates the accumulation of the toxins in the kidney and, therefore, it is not recommended (Danel et al., 2001; Schumacher and Hoiland, 1983).

Complete recovery of renal function is attained only in 30% of the poisoned patients, with the majority of patients presenting a healing of fibrosis and a variable loss of renal function. Long-term renal replacement therapy is needed in 20-40% of patients (Bouget et al., 1990; Kilner et al., 1999). Therefore, renal transplantation has been considered, but should not be performed too early in the course of illness. The median time for transplant are 9-10 months after presentation, wherein appears safe without risk of further toxicity from OR (Danel et al., 2001; Holmdahl and Blohme, 1995).

Part II

Aims

2. AIMS

OR is a potent toxin produced by *Cortinarius* mushrooms which grow throughout in Europe and parts of North America. *Cortinarius orellanus* and *Cortinarius speciossimus* are the major OR-containing mushrooms which are responsible for causing human intoxication. OR poisoning is characterized by severe oliguric acute renal failure, with a mortality rate of 10-30%. Chemically, OR resembles the bipyridyl herbicides diquat and PQ. The mechanism of toxicity of these compounds implicates oxidation/reduction of chain reactions, with ultimate formation of free radicals and lowering of NADPH concentration.

Given the similarities identified between OR and PQ, we can suspect that the antidotal compound used in PQ poisoning, LAS, has a great potential to be successfully used in orellanine poisoning.

Considering the absence of a specific antidote for OR poisoning, two types of *in vivo* studies were performed in order to prove the efficacy of LAS against OR intoxication: a short-term study (sacrifice time at 24 hours) and a survival study. The first study was conducted to histological damage, biochemical analysis in plasma and urine, oxidative stress biomarkers (lipid peroxidation and GSH/GSSG) and total RNA quantification. The subsequent survival study aimed to evaluate the long-term effectiveness of LAS. Another aim of this dissertation was to evaluate the toxic effects of OR in the target organs and to evaluate possible poisoning signs and symptoms not previously described.

Part III

Materials and Methods

3. MATERIALS AND METHODS

3.1 Chemicals and drugs

OR was purchased from Apollo Scientific (Denton, Manchester). LAS was obtained from. Xylazine and ketamine were purchased from Bayer (Carnaxide, Portugal) and Vetoquinol (Barcarena, Portugal), respectively. RNALater, TRI Reagent, RNASE free water, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), Nicotinamide adenine dinucleotide phosphate (NADPH), glutathione reductase, L-glutathione oxidized, L-glutathione reduced, 2-vinylpyridine, RNAlater, 2-thiobarbituric acid (TBA), malondialdehyde (MDA) and X-triton were purchased from Sigma-Aldrich (St. Louis, USA). Percholoric acid ($HClO_4$), Potassium bicarbonate ($KHCO_3$), Ethylenediaminetetraacetic acid (EDTA), Disodium phosphate (Na_2HPO_4), sodium hydroxide (NaOH), Monosodium phosphate (NaH_2PO_4), potassium dihydrogen phosphate (KH_2PO_4) and paraffin were obtained from Merck (Darmstadt, Germany). Sodium chloride (NaCl) was purchased from VWR (Leuven, Belgium), glycoblue from Ambion (Lithuania), isopropyl alcohol (C_3H_8O) from Panreac (Barcelona, Spain), chloroform ($CHCl_3$) from Fisher Scientific (Loughborough, UK), buffered formalin from Klinipath (Netherlands), ethanol from Panreac AppliChem (Darmstadt, Germany), xylene from BDH – Prolabo, VWR International (Ireland) and silane from Nuova Aptaca (Canelli, Italy).

N-acetyl-β-glucosaminidase (NAG) reagents were obtained from Diazyme Europe GmbH. The reagents Albumine, total protein (TP), cholesterol (Chol), high-density lipoprotein (HDL), creatinine, uric acid (UA), triglycerides (TG), alanine aminotransferase (ALT), glucose, amylase, low-density lipoprotein (LDL), lactate dehydrogenase (LDH), creatinine kinase (CK), creatinine kinase MB (CK-MB), phosphate, magnesium (Mg), calcium (Ca), iron, aspartate aminotransferase (AST), chloride, Pseudocholinesterase (PchE), lactate and γ-glutamyl-transpeptidase (GGT) were purchased from PVL LTD (Lisbon, Portugal).

3.2 Animals and experimental design

In the present work, two *in vivo* studies were performed to evaluate the potential protective effect of LAS against OR toxicity: a short-term study (24 hours) and a survival study (5 months). The short-term study was performed using adult male Wistar rats obtained from Charles River S.A. (Barcelona, Spain), with a mean weight of 150-200 g. Adult male CD-1 mice (Harlan, Udine, Italy), weighing 30-40 g, were used in all experiments of the survival rate study. In both studies, before starting the experiments, animals were maintained under a controlled environment (12/12 h light/darkness, 22±2°C room temperature, 50-60% humidity) for at least 1 week (quarantine) and 5 months, for both the short and the survival studies, respectively. Animals were allowed access to tap water and rat chow *ad libitum* during these periods.

All procedures were carried out to provide an appropriate animal care, minimizing their suffering.

3.2.1 Short-term study

Our work started with a short-term (24 h) study to evaluate the effectiveness of LAS in protecting lung, liver, kidney, heart and spleen against the toxicity of OR. In order to create a real scenario of intoxication, since intoxicated people only arrive to emergency rooms hours or even days after mushrooms ingestion, LAS was only administered to animals 4 h after OR administration. After the quarantine period, 20 animals were randomly divided in four groups of 5 animals each that were treated as follows: (1) control group, animals subjected to two 0.9% saline solution (0 and 4 h); (2) OR group, animals exposed to one dose of OR (15 mg/kg) followed by one 0.9% saline solution 4 h after OR administration; (3) LAS group, animals exposed to a 0.9% saline solution followed by one dose of LAS (200 mg/kg) 4 h after saline administration; and (4) OR + LAS group, animals exposed to one dose of OR (15 mg/kg) followed by one dose of LAS (200 mg/kg) 4 h later. All drugs were given intraperitoneally (i.p.). The dose of OR used was chosen based on previously reported data indicating to be the lethal dose 50 (LD_{50}) in mouse (Prast et al., 1988). The LAS dose was selected according to the study performed by Dinis-Oliveira and colleagues (Dinis-Oliveira et al., 2007).

Each animal was individually housed in a metabolic cage where it was kept during the whole time of experiment (24 h). Animals were fasted during the entire experimental period but water was given *ad libitum*. Twenty-four hours after the last administration, all

animals were anesthetized with xylazine/ketamine i.p. (10 and 100 mg/kg, respectively). Animals were placed in the *decubito supine* position and the thorax was opened by two lateral transversal incisions and one central longitudinal incision to expose the aorta artery. Blood, urine and solid tissue samples (liver, kidneys, lungs, heart and spleen) were collected for subsequent biochemical analysis.

3.2.2 Survival rate study

For the evaluation of survival rate, 6 animals were randomly divided into two groups of 3 animals each. The two groups were treated as follows: (1) OR group, animals exposed to one dose of OR (3.5 mg/kg i.p.) followed by one 0.9% saline solution i.p. 4 h after OR administration; (2) OR+LAS group, animals exposed to one dose of OR (3.5 mg/kg i.p.) followed by one dose of LAS (200 mg/kg i.p.) 4 h later. The dose of OR administered was chosen based on previously reported data indicating to cause a deteriorated renal function and an increased oxidative damage in rats kidneys (Nilsson et al., 2008).

Body weight, motor activity, dyspnea, piloerection, dehydration, bleeding, color of mucous, abdominal pain, signs and changes in behavior, if present, were noted and recorded. All mentioned parameters were monitored for 5 months.

3.3 Short-term study

3.3.1 Blood collection and biochemical analysis

In the short-term study, the blood was collected with a heparinized needle from the inferior vena cava and was placed into a heparinized containing tube. Blood samples were subjected to water bath at 37°C for 2-3 min and were then centrifuged (5000g, 4°C, for 4 min). The plasma supernatant was collected into tubes and stored at -80°C until determination of albumin, total protein, cholesterol, high-density lipoprotein (HDL), creatinine, uric acid, triglycerides (TG), alanine aminotransferease (ALT), glucose, amylase, low-density lipoprotein (LDL), lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase isoform MB (CK-MB), phosphate, magnesium, calcium, iron, aspartate aminotransferase (AST), chloride, pseudocholinesterase (PchE) and lactate. Plasma biochemical parameters were measured on an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.).

3.3.2 Urine collection

Urine samples were centrifuged (13000g, 4°C, for 10 min) and stored (-80°C) until determination of urea, creatinine, total protein and uric acid. Urinary biochemical parameters were measured on an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.). NAG and clearance of creatinine were determined. Clearance of creatinine was calculated as follows: $\frac{Urinary\ creatinine \times urine\ volume\ 24\ hours}{Plasmatic\ creatinine \times 1440 \times body\ weight}$ (mL/min/Kg).

3.3.3 Assessment of NAG activity

Urinary NAG activity was assayed according to the kit DZ062A-K Diazyme N-Acetyl-β-D-glucosaminidase (NAG) assay (USA). The assay principle was determined by the kinetic spectrophotometric measurement of the rate of appearance of the reaction product of a direct enzymatic cleavage of a synthetic substrate by NAG. NAG hydrolyzes 2-methoxy-4-(2'nitrovinyl)-phenyl 2-acetamido- 2-deoxy-β-D-glucopyranoside (MNP-GlcNAc) to 2-methoxy-4-(2'-nitrovinyl)-phenol. Briefly, the assay mixture consisted of 10µL sample, calibrator or standard and 150µL reagent mix (MNP-G1cNA (dissolved in hydrochloric acid) citric acid (dissolved in potassium phosphate, pH 4.7) 5:1 (v/v)). The samples were incubated at 37°C for 5 min in plate reader (BioTek Instruments, Vermont, US). The product formation was detected by development of color at 505 nm upon addition of 50µL reagent 3 (sodium carbonate buffer, pH 10). Urinary NAG activity was assayed in triplicated and results were expressed in U/L.

3.3.4 Collection and processing organs for biochemical analysis

After blood collection, lung, liver, kidney, heart and spleen were removed, weighted and processed as follows: (i) slices of lung, liver, kidney, heart and spleen were kept in RNAlater and stored at -80°C for future total RNAquantification and quantitative PCR analysis; (ii) segments of lung, liver, kidney, heart and spleen were placed in 4% buffered formalin and used for histological and immunohistochemistry analysis; (iii) segments of lung, liver, kidney, heart and spleen were homogenized (1:4 m/v) with an Ultra-Turrax®

homogenizer in cold phosphate buffer [(2.67 g Na_2HPO_4, 1.7025 g KH_2PO_4 and 0.5 mL Triton X-100), pH 7.4] and centrifuged (3000g, 4°C, for 10 min). Aliquots of the resulting supernatants were transferred to eppendorfs and centrifuged again (13000 g, 4°C, for 10 min). The resulting supernatants were divided in aliquots and stored (-80°C) until posterior activity and biochemical parameters quantification (albumin, TP, chol, HDL, glucose, creatinine, uric acid, TG, ALT, amylase, GGT, LDL, LDH, CK, CK-MB, phosphate, Mg, Ca, iron, AST, chloride and lactate); (iv) perchloric acid (10%) was added to the resulting supernatant described above in the proportion of 1:1 and then centrifuged at 13000 g, 4ºC, for 10 min. The resulting supernatant was stored (−80°C) until quantification of total glutathione (GSHt), reduced glutathione (GSH), oxidized glutathione (GSSG) and lipid peroxidation.

3.3.5 Quantification of GSHt, GSH and GSSG

The GSHt and GSSG contents in lung, liver, kidney, heart and spleen perchloric acid supernatant were determined by the DTNB-GSSG reductase recycling assay, performed in triplicate, as described before (Pontes et al., 2008), with some modifications. Briefly, the thawed acidic supernatant was neutralized with equal volume (200µl) of 0.76 M $KHCO_3$ and centrifuged for 10 min at 13000g (4°C). For measurement of GSHt, 100 µL/well of the neutralized supernatants, standards or blank were added in triplicate to 96-well microtiter plates, followed by 65 µL/well of freshly prepared reagent containing 0.24 mM NADPH and 0.7 mM DTNB in phosphate buffer and deionized water. Plates were then incubated at 30°C in plate reader (BioTek Instruments, Vermont, US), for 15 min prior to the addition of 40 µL/well of a 10IU/mL glutathione reductase solution in phosphate buffer. The stoichiometric formation of 5-thio-2-nitrobenzoic acid was followed for 3 min at 415 nm and compared to a standard curve. Standards were made in perchloric acid solution 5%, being the range of concentrations used between 0 and 15 nmol/mL.

To determine GSSG levels, before the neutralization 10 µL of 2-vinylpyridine was added to the samples homogenates, standards and blanks and mixed continuously for 1h, on ice, to block the reduce glutathione (GSH). GSSG was then measured as described above for GSHt. The GSSG standard solutions were also made in 5% perchloric acid with concentrations that ranged between 0 and 8 nmol/mL. The molar GSH levels were calculated by subtracting the GSSG content for the total glutathione content ($GSH = GSHt - 2 \times GSSG$). Results are expressed in nmol of GSH or GSSG per mg of protein.

3.3.6 Evaluation of lipid peroxidation

Lung, liver, kidney, heart and spleen lipid peroxidation were measured by the thiobarbituric acid reactive substances (TBARS) assay methodology. TBARS assay involves the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) under strong acidic condition and heating, leading to the formation of pink colored products which can be measured by fluorometric methods (Niki, 2014). It was used the improved assay method described by (Buege and Aust, 1978), with slight alterations. Briefly, the assay mixture consisted of 200µL sample, blank or standard and 200µL TBA (dissolved in distilled water). The samples were vortex mixed and incubated at 80°C for 60 min in water bath. After incubation, mixture was cooled in water vortex mixed. Fluorescence were read (BioTek Instruments, Vermont, US) at $\lambda_{ex}/_{em}$ = 530-590 nm. The MDA standard solutions were also made in 5% perchloric acid with concentrations that ranged between 0 and 30 µmol/mL. The results were expressed as nmol of malondialdehyde (MDA) equivalents/mg protein.

3.3.7 RNA extraction and real-time PCR

Total RNA isolation was performed by adding 800µL of TRI Reagent to lung, liver, kidney, heart and spleen samples. All specimens were homogenized by mechanical disruption using the Ultra-Turrax® instrument and total RNA was extracted in RNAse-free environment. The homogenized samples were incubated for 5 min at room temperature. 160µL chloroform was added per 800µL of TRI Reagent. The samples were mixed vigorously and then centrifuged at 12,000 x g for 10 min at 2-8 °C. Centrifugation separated the biphasic mixtures into the lower red, phenol-chloroform phase and the upper colorless, aqueous phase. The RNA was precipitated from aqueous phase by mixing with 800µL of isopropanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 2-8 °C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. The pellet was air dried and dissolved in water RNAse free. The RNA concentration was determined by OD260 measurement using a NanoDropH ND-1000 Spectrophotometer (NanoDrop Technologies, USA), and the purity of the total RNA extracted was assessed by measuring the absorbance at 230 and 280 nm. 200 ng of total RNA was reverse-transcribed using the iScript Select cDNA Synthesis it (Bio-Rad, Hercules, California, USA) according to the

manufacturer's protocol. All cDNA samples were stored at -20 °C until quantitative real-time PCR (qPCR) analysis. qPCR was performed in iQ™ 5 Real-Time PCR detection System (Bio-Rad, Hercules, California, USA) in 96-well plates with a reaction volume of 20 µL and runs up to 40 cycles using iQ™ SYBER® Green Supermix. The final PCR reaction mixture of 10 µL contained 0.25 µL of cDNA samples, 5 µL of iQ™ SYBER® Green Supermix, 0.25 µL of each primer and 4.25 µL of RNase-free water. The cycling conditions were set as follows: Taq DNA polymerase activation at 95 °C for 3 min, amplification steps: desnaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 72 °C for 15 s with fluorescence acquisition. Two highly stable reference genes for RNA polymerase II (RNAPII) were chosen (βeta-actin, GAPDH) as well as two ribosomal 18S and 28S genes transcribed by RNA polymerase I (RNAP I). All cDNA samples were measured in duplicate, and the relative transcript levels were quantified by the threshold cycle (Ct) value. All primers were designed using the Beacon Designer Software (version 7.2, PREMIER Biosoft Internation, Palo Alto, CA, USA).

3.3.8 Histological analysis

Histology sampling protocol was designed with the goal of ensuring the validity of the estimators to minimizing the variance contribution from sampling variation. The spleen was cut in the midsagittal plane in two halves. The heart, kidneys and lungs were divided in two equal parts: the cranial and caudal parts. Each organ half was then randomly selected for histological analysis. Liver was cut into ≈ 4 mm thick slabs and then a systematic selection was carried out and sampled pieces were processed for light microscopy. Organs fragments were fixed in 4% buffered formalin and processed for histological examination by light microscopy. After fixation (24 hours), the tissues were dehydrated through a series of graded ethanol solutions (70-99.8%), cleared in xylene, and impregnated and embedded in paraffin. Each organ was sectioned (Microtome - Leica RM 2255, Germany) into thin sections (5 µm thick). For improving section adhesion, the sampled sections were mounted in silane coated microscope slides. The sections were then stained with haematoxylin-eosin (H&E) before being coversliped.

3.4 Statistical analysis

All data obtained were expressed as mean ± SEM (standard error of the mean). All statistical analysis was performed using GraphPad Prism® (version 6.01, GraphPad Software, San Diego, California, USA). The Shapiro-Wilk test was performed to check normality of the data. Statistical comparisons were done using the one-way ANOVA (in case of normal distribution) followed by the Bonferroni post hoc test or Kruskal-Wallis (in case of not normal distribution) followed by the Dunn's post hoc test. *P* values lower than 0.05 were considered statistically significant.

Part IV

Results

4. RESULTS

4.1 Short-term study

4.1.1 Organs weight

As an indirect measure of organ damage, each organ (kidney, liver, lung, spleen and heart) had their weight registered and the weight ratio of each organ was taken to body weight. No significant differences were observed between OR group and the control group concerning the kidney weight /body weight ratio, liver weight /body weight ratio, lung weight /body weight ratio, spleen weight /body weight ratio and heart weight /body weight ratio (Table 4).

Table 4 – Kidney weight/body weight ratio, liver weight /body weight ratio, lung weight /body weight ratio, spleen weight /body weight ratio and heart weight /body weight ratio of control, OR, LAS and OR+LAS groups.

	Control	OR	LAS	OR + LAS
Kidney	7.15±0.30	7.37±0.15	7.24±0.27	7.10±0.21
Liver	28.52±1.07	28.99±0.67	28.24±0.68	28.43±0.56
Lung	4.78±0.17	4.78±0.19	4.69±0.18	4.54±0.14
Spleen	2.20±0.19	2.28±0.14	2.48±0.20	2.15±0.048
Heart	3.29±0.14	3.38±0.10	3.04±0.03	3.08±0.19

Values are given as mean±SEM (*n* = 5).

4.1.2 Biochemical analysis in plasma and urine

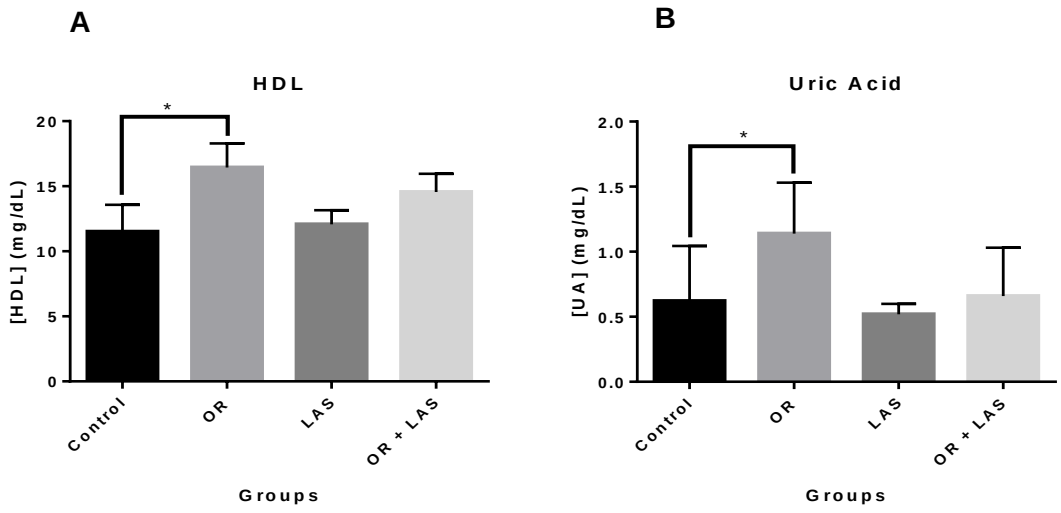
Plasma biochemistry quantified 24h after intoxication only showed slight variations (Table 5). HDL and uric acid levels were significantly increased in the OR group (16.44±1.85, *p<0.05,* and 1.14±0.39 mg/dL, *p<0.05,* for HDL and uric acid, respectively) as compared with control group (9.48±0.60 and 0.20±0.07 mg/dL for HDL and uric acid, respectively). While this effect was reverted in the OR+LAS group (14.56± and 0.30±0.12 mg/dL for HDL and uric acid, respectively) (Figure 6A and 6B). On the other hand, amylase, CK and PChE levels were significantly increased in the OR+LAS group (399.00±16.39, *p<0.05*, 2060.00±282.90, *p<0.05,* and 492.90±58.96, *p<0.05,* U/L for amylase, CK, PChe respectively) compared to control group (325.10±16.84, 656.00±147.80 and 227.40±48.57) (Figure 6C, 6D and 6E). The calcium levels were significantly decreased in the OR (7.97±0.12, *p<0.05*) and OR+LAS (7.05±0.28, *p<0.01*)

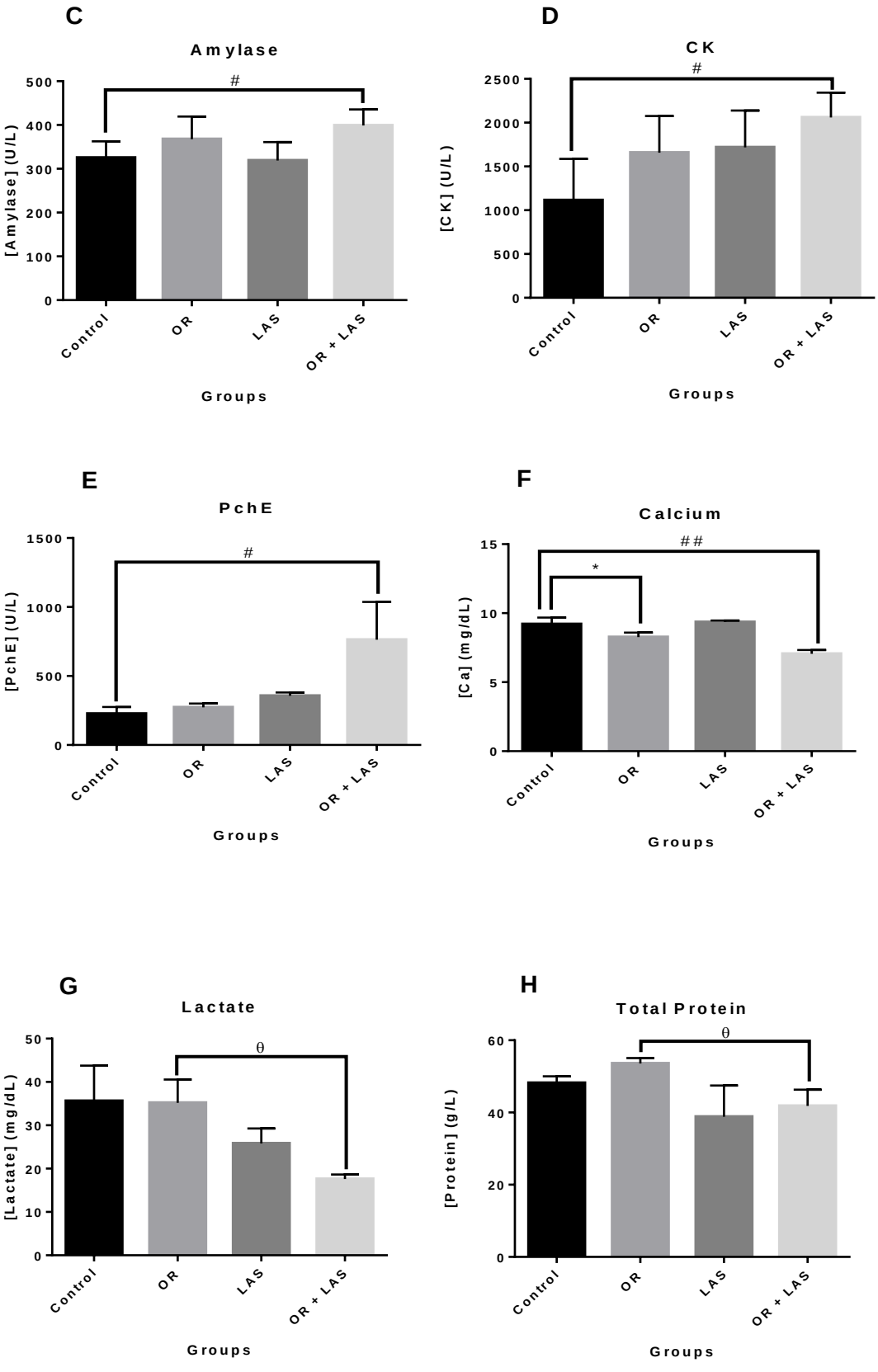
group between control group (9.21±0.47) (Figure 6F). There was a statistically significant increase in lactate and total protein in OR-treated animals (35.20±5.38, *p<0.05,* and 53.59±1.51, *p<0.05*) compared to OR+LAS group (17.60±1.03 and 48.19±1.86) (Figure 6G and 6H). The chloride levels significantly decreased in OR group (93.84±0.31, *p<0.05*) compared with OR+LAS-treated animals (99.12±0.31) (Figure 6I).

Table 5 – Plasma biochemistry following 24h of intoxication.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	3.09 ±0.17	3.42±0.01	3.07±0.07	3.37±0.215
TP (g/L)	48.19±1.86	53.59±1.51$^{\theta}$	47.44±1.29	41.81±4.52
Chol(mg/dL)	32.18±2.15	54.86±5.80	47.90±5.59	48.38±11.79
HDL (mg/dL)	9.48±0.60*	16.44±1.85*	2.08±1.08	14.56±1.40
LDL (mg/dL)	14.03±2.97	15.16±1.02	18.66±4.86	12.68±0.88
Glucose (mg/dL)	85.96±11.20	98.60±10.68	98.60±5.20	110.80±8.97
Creatinine (mg/dL)	0.04±0.02	0.003±0.003	0±0	0±0
UA (mg/dL)	0.20±0.07	1.14±0.39*	0.52±0.08	0.30±0.16
TG (mg/dL)	3.28±2.02	4.18±2.73	13.38±5.12	12.62±6.22
Amylase (U/L)	325.10±16.84	367.70±23.06	319.10±18.73	399.00±16.39$^{\#}$
ALT (U/L)	0.38±0.23	0.08±0.08	0.08±0.06	0.40±0.25
LDH (U/L)	455.20±103.80	557.70±86.49	644.90±90.29	759.40±129.20
AST (U/L)	115.70±12.42	168.80±24.05	124.40±6.95	149.50±13.26
CK (U/L)	656.00±147.80	1657.00±418.20	1718.0±419.30	2060.00±282.90$^{\#}$
CK-MB (U/L)	127.00±26.63	126.2±22.49	153.60±24.71	204.30±31.94
Chloride (mg/dL)	95.88±2.67	93.84±0.31$^{\theta}$	95.95±2.98	99.12±0.31
Phos (mg/dL)	9.72±0.44	9.03±0.55	9.15±0.811	9.28±0.64
Mg (mg/dL)	3.15±0.30	3.18±0.35	3.08±0.34	3.21±0.36
Ca (mg/dL)	9.21±0.47	7.97±0.12*	9.35±0.11	7.05±0.28$^{\#\#}$
Iron (mg/dL)	289.70±44.86	210.70±82.99	371.10±109.20	630.00±328.30
PChe (U/L)	227.40±48.57	273.40±27.79	356.90±24.25	492.90±58.96$^{\#}$
Lactate (mg/dL)	27.50±1.32	35.20±5.38$^{\theta}$	25.80±3.46	17.60±1.03

Values are given as mean±SEM (n = 5); * p < 0.05 OR *vs*. Control; # p < 0.05 OR+LAS *vs*. Control; $^{\theta}$ p < 0.05 OR *vs* OR+LAS; ## p < 0.01 OR+LAS *vs*. Control.

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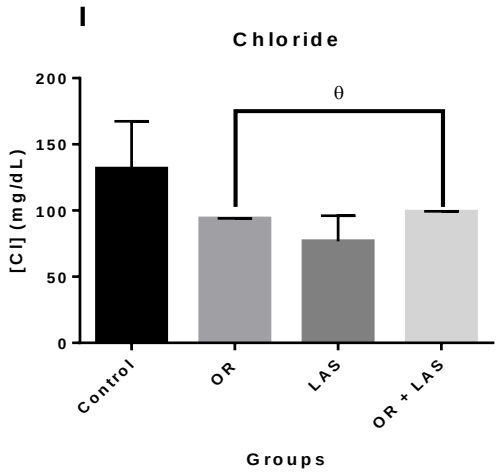

Figure 6. Plasmatic levels of (A) HDL, (B) uric acid, (C) amylase, (D) CK, (E) PChE, (F) Calcium , (G) lactate , (H) total protein and (I) chloride in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparison were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (* $p < 0.05$ OR vs. Control; # $p < 0.05$ OR+LAS vs. Control; $^{\theta}$ $p < 0.05$ OR vs OR+LAS; ## $p < 0.01$ OR+LAS *vs*. Control).

As shown in Figure 7A, animals from group OR+LAS exhibited an increase of NAG levels in urine compared to control group (15.57±3.88 vs 7.38±1.33 U/L from NAG). However, this parameter was kept near to control levels in the OR group (7.76±1.54 U/L from NAG). Analogous results were obtained for TP (3.66±0.32, *p<0.01,* vs 1.04± 0.26g/L for total protein), while for the clearance of creatinine it was observed, in OR group, an increase compared to control and OR+LAS groups (47.53±33.19 vs 6.18±5.99 vs 0) (Figure 7B and 7C). The animals exposed to OR+LAS had lower levels of urea compared to the control group (115.60±27.60, *p<0.05,* vs 220.60±19.97 mg/dL for urea) (Figure 7D).

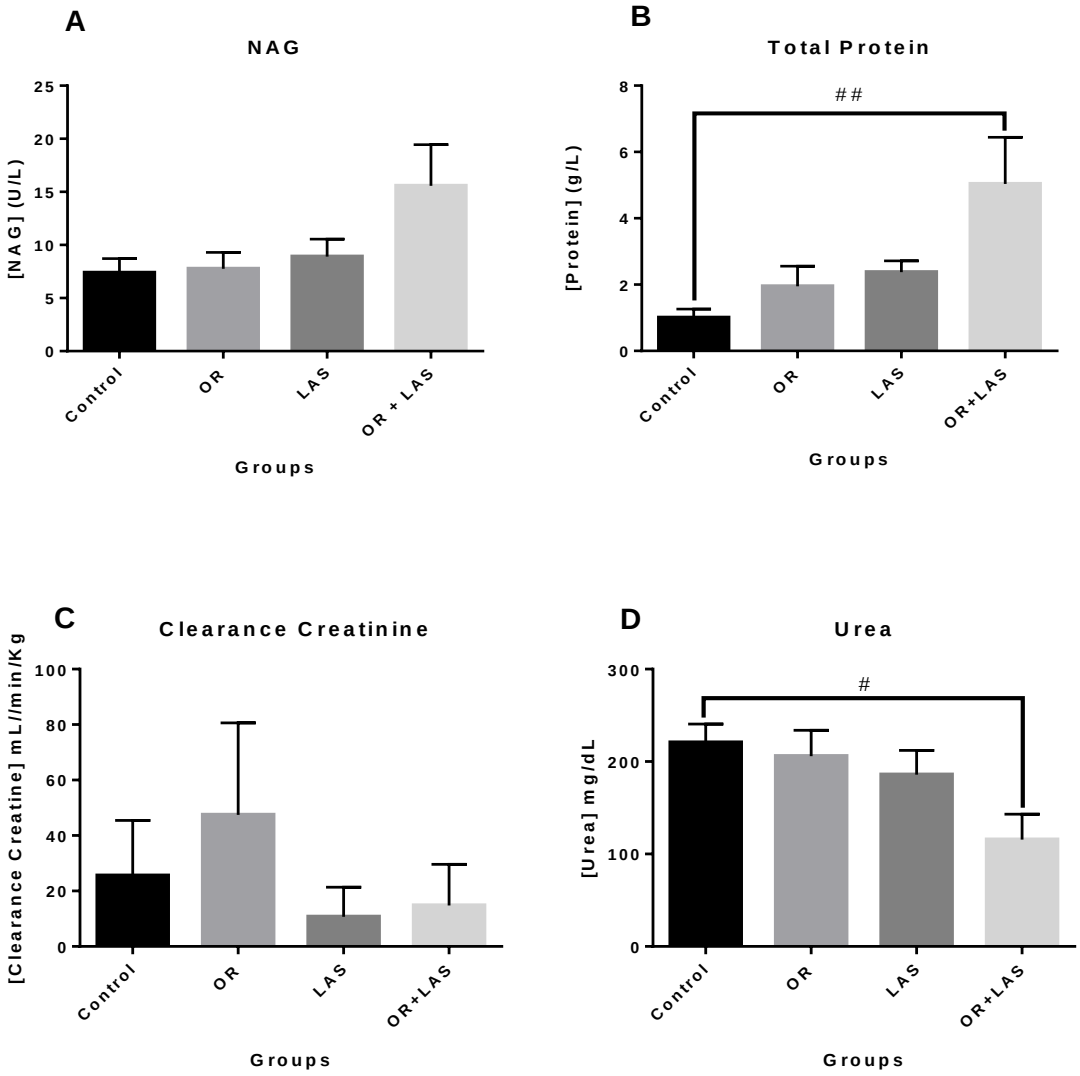

Figure 7. Urine levels of (A) NAG, (B) total protein, (C) clearance of creatinine and (D) urea in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (# $p < 0.05$ OR+LAS vs. Control; ## $p < 0.01$ OR+LAS *vs*. Control).

Table 6 – Urinary biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	0 ± 0	0±0	0±0	0±0
TP (g/L)	1.00±0.26	1.95±0.60	2.38±0.34	3.66±0.32$^{##}$
Creatinine(mg/dL)	22.98±6.52	46.05±11.91	31.73±4.72	61.04±20.15

UA (mg/dL)	9.84±1.15	8.54±3.61	8.66±3.79	14.98±1.10
Urea (mg/dL)	220.60±19.97	205.90±28	185.80±26.33	115.60±27.60#
NAG (U/L)	7.38±1.33	7.76±1.54	8.91±1.64	15.57±3.88
Clearance Creatinine (mL/min/Kg)	6.18±5.99	47.43±33.19	0±0	0±0

Values are given as mean±SEM (*n* = 5); # *p* < 0.05 OR+LAS *vs*. Control*; ## p* < 0.01 OR+LAS *vs*. Control.

4.1.3 Biochemical analysis in organs

The mechanism of toxicity involved in the poisoning by orellanine is not yet fully understood. Therefore, we evaluated the clinical biochemistry of the kidney, liver, lung, spleen and heart (Attachements A1, A2, A3, A4 and A5).

A significant increase was observed in the lung HDL levels of the OR+LAS group (3.12±0.77, *p<0.05*) as compared to the control group (0.80±0.34) (Figure 8).

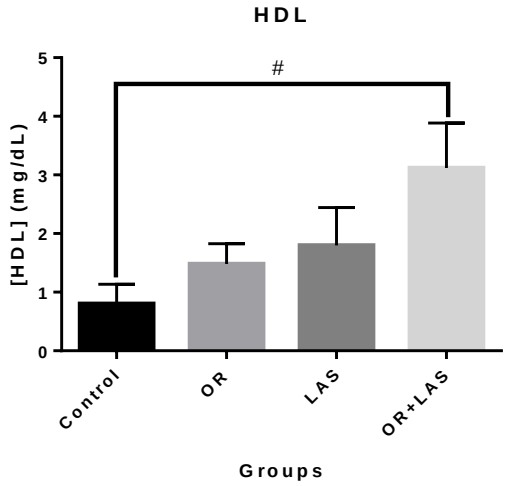

Figure 8. Lung levels of HDL in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (# p < 0.05 OR+LAS vs. Control).

As shown in Fig 9, heart albumin and phosphate levels were significantly decreased in the OR+LAS group (0.05±0.14 g/dL and 27.58±1.49 mg/dL, *p<0.05*) compared to control and OR groups, respectively (0.10±0.03 g/dL and 32.93±0.67 mg/dL).

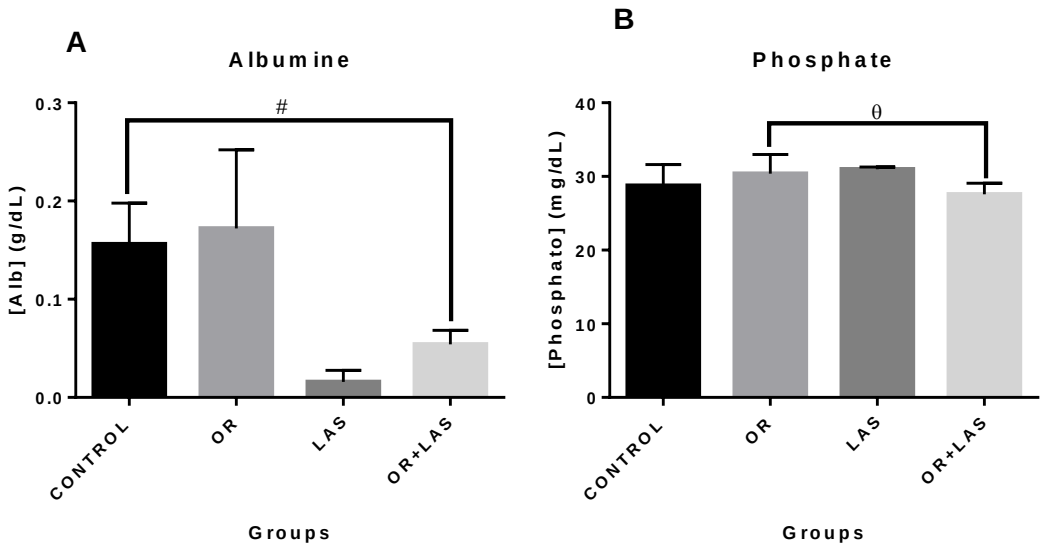

Figure 9. Heart levels of (A) albumin and (B) phosphate in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparison were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (# $p < 0.05$ OR+LAS vs. Control; $^{\theta}$ $p < 0.05$ OR vs OR+LAS).

4.1.4 Oxidative stress biomarkers

Due to the fact OR produces ROS during the redox cycling, oxidative stress and inflammatory biomarkers were also evaluated.

As shown in Figure 10, kidney GSH levels decreased in OR+LAS group (0.94±0.38 nmol/mg protein) when compared to control group (1.16±0.22 nmol/mg protein), although no statistical significance was reached. A significant increase was observed in the GSSG levels of the OR+LAS group (0.76±0.24 nmol/mg protein, *p<0.01*). In accordance, the GSH/GSSG ratio suffered a significant reduction in the OR+LAS group compared to the control group (0.58±0.23 *vs* 13.00±2.59, *p<0.05*).

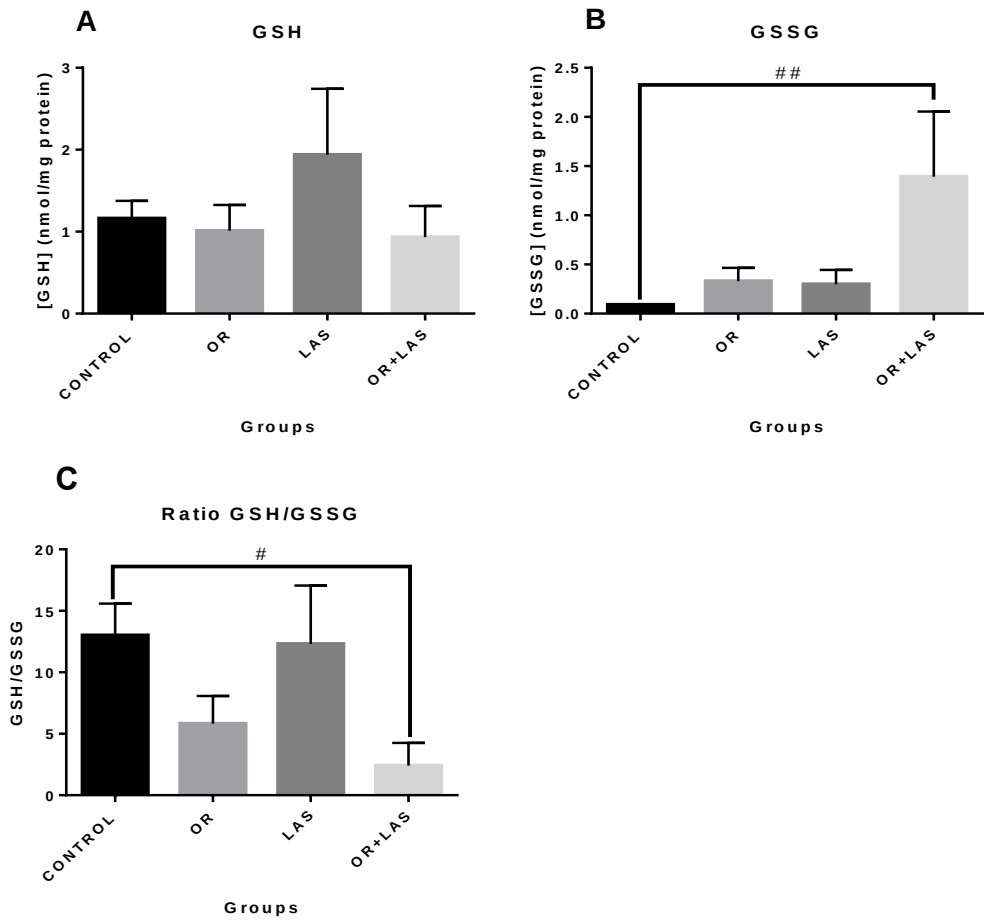

Figure 10. Kidney levels of (A) GSH, (B) GSSG and (C) GSH/ GSSG ratio in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision was performed using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (# p < 0.05 OR+LAS vs. Control; ## *p* < 0.01 OR+LAS *vs*. Control).

No significant changes in the liver GSH levels were observed between control, OR and OR+LAS group (Figure 11). It was observed only a tendency to decrease in both OR (13.13±2.33 nmol/mg protein) and OR+LAS (15.53±3.43 nmol/mg protein) groups when compared to control group (19.52±2.31 nmol/mg protein). A significant increase was observed in the GSSG levels of the OR+LAS group (14.93±1.60 nmol/mg protein, *p<0.0001*) compared to control (1.476±0.3351 nmol/mg protein) and OR (5.07±1.22 nmol/mg protein, *p<0.05*) groups. In conformity, the GSH/GSSG ratio suffered a significant reduction in the OR+LAS group (1.14± 0.31, *p<0.01*) compared to the control group (11.28±1.89).

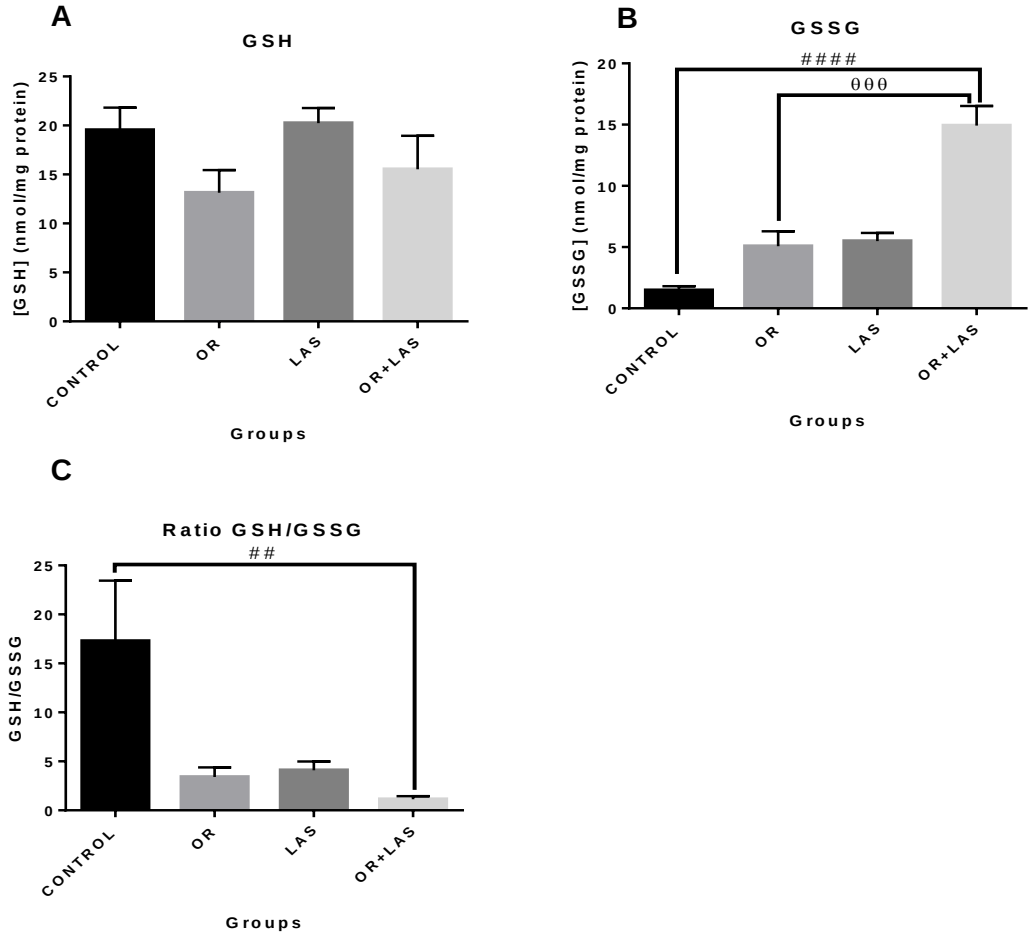

Figure 11. Liver levels of (A) GSH, (B) GSSG and (C) GSH/ GSSG ratio in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (## $p < 0.01$ OR+LAS *vs*. Control; θθθ $p < 0.001$ OR *vs* OR+LAS; #### $p < 0.0001$ OR+LAS *vs*. Control).

There were no significant changes in lung GSH levels, but it was observed a significant increase in the GSSG levels of the OR group when compared to control group (5.73±0.90 *vs* 1.17±0.40 nmol/mg protein, *p<0.05*). In accordance, the GSH/GSSG ratio suffered a significant reduction in the OR group compared to control group (1.80±0.39 *vs* 6.67±1.05, *p<0.05*) (Figure 12).

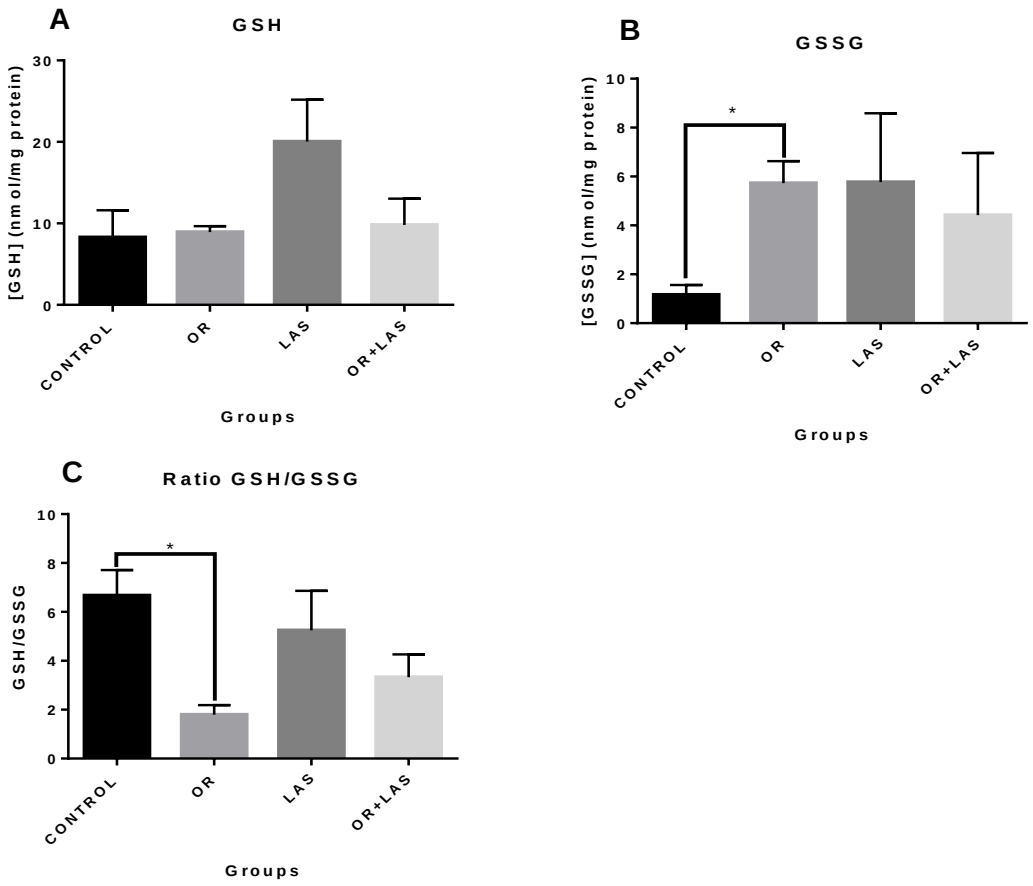

Figure 12. Lung levels of (A) GSH, (B) GSSG and (C) GSH/ GSSG ratio in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision were using Krusal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (* $p < 0.05$ OR vs. Control).

The amounts of GSH in its reduced state and GSSG were measured in the spleen but no significant differences were observed. Observing the results, the GSH/GSSG ratio suffered a significant reduction in both OR and OR+LAS (1.95±0.63 *(p<0.01)* and 3.14±0.62 *(p<0.05),* respectively) groups compared to control group (6.27±1.11) (Figure 13).

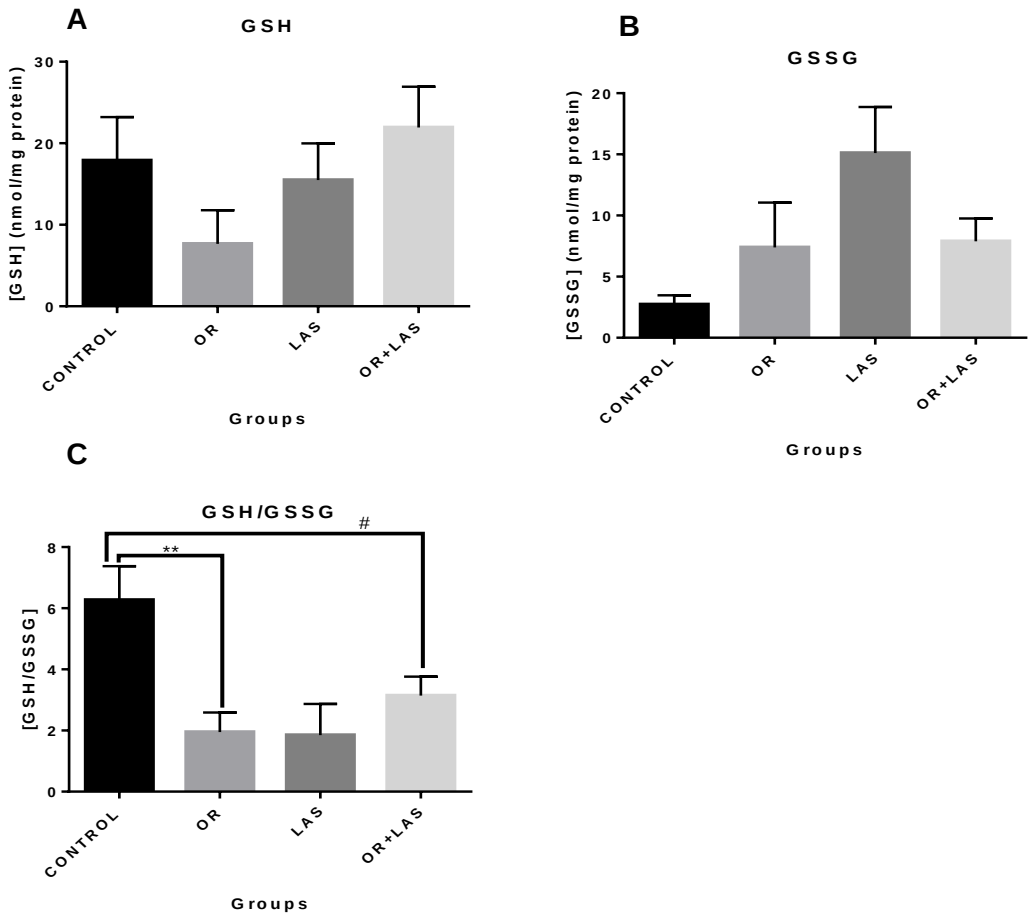

Figure 13. Spleen levels of (A) GSH, (B) GSSG and (C) GSH/ GSSG ratio in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparison were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (#p < 0.05 OR+LAS vs. Control;**p < 0.01 OR *vs*. Control).

In Figure 14, the levels of GSH, GSSH and GSH/GSSG ratio in the heart of the animals can be observed. There were no significant differences of all these parameters between the different groups.

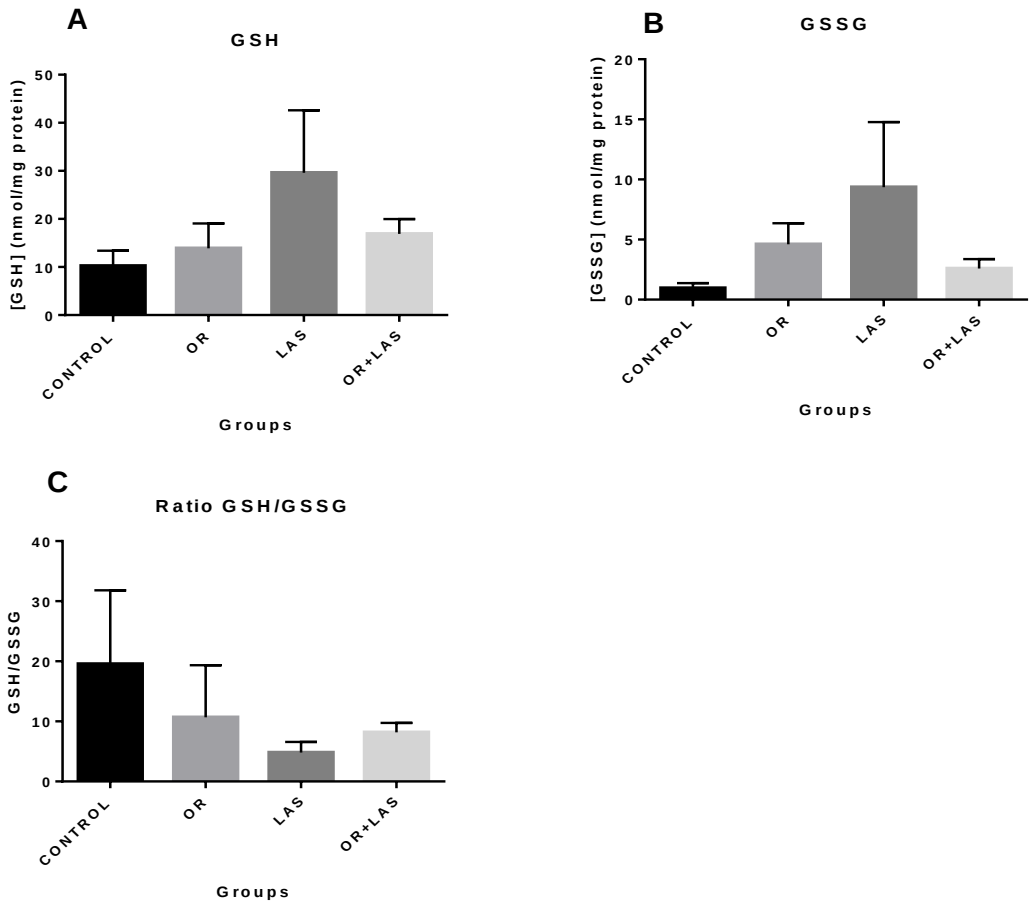

Figure 14. Heart levels of (A) GSH, (B) GSSG and (C) GSH/ GSSG ratio in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparison were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test*

Lipid peroxidation was assessed by means of MDA equivalents. The levels of MDA equivalents in the kidney, liver, lung, spleen and heart were also determined. As present in table 7, there were no significant alterations in the MDA equivalents levels in all considered groups.

Table 7 – Amount of malondialdehyde (MDA nmol/mf of protein) in the control, OR, LAS and OR+LAS groups.

	Control	OR	LAS	OR + LAS
Kidney	2676.0±751.90	1187.00±234.60	2723.00±647.70	1989.00±717.20
Liver	408.70±60.74	407.60±68.70	391.60±70.66	327.70±81.83
Lung	1077.0±405.60	1088.00±309.50	1067.00±123.1	1757.00±649.60
Spleen	2762±852.60	2316±837.3	1688±515.6	3023±770.3
Heart	1322.0±429.80	1385.0±468.00	2174.0±488.80	1620.0±4462.00

Values are given as mean±SEM (*n* = 5).

4.1.5 Histology

4.1.5.1 Kidney

The examination of the hemisected kidney section of rats of control group revealed the normal morphologic features of the species (Figure 15). The cortex consists of renal corpuscles along with the convoluted tubules and straight tubules of the nephron, the collecting tubules, collecting ducts, and an extensive vascular supply.

Morphologic alterations were observed in all groups being more numerous and more diverse in the kidney of rats exposed to OR+LAS (Figure 16C and F), followed by OR (Figure 16B and E) and LAS (Figure 16 A and D) groups. The tubules were first and foremost damaged by OR, probably as a result of a hypovolemic shock. It was observed both tubular ectasia and tubular proteinosis, and epithelial cells of the affected tubules exhibited marked cytoplasmic degenerative changes or necrosis and apoptosis. As previously mentioned, the volume of the affected tubules was apparently higher in the rats of OR+LAS group.

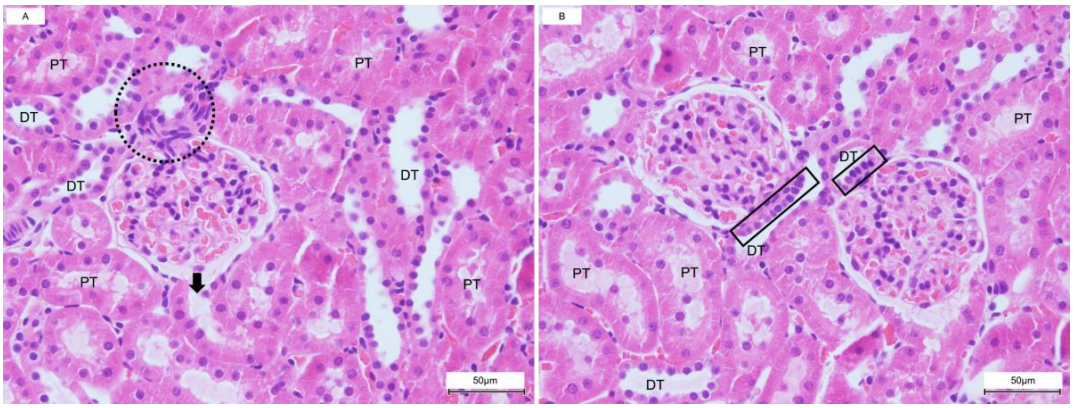

Figure 15. Representative kidney image from rat of Control group. Renal corpuscles consist of the glomerulus, surrounded by a double-layered epithelial cup, the renal or Bowman's capsule. The glomerular capillaries are supplied by an afferent arteriole and

are drained by an efferent arteriole that then branches, forming a new capillary network to supply the kidney tubules. The site where the afferent and efferent arterioles penetrate and exit from the parietal layer of Bowman's capsule is called the vascular pole (marked with the circle in A). Opposite this site is the urinary pole of the renal corpuscle, where the proximal convoluted tubule begins (thicker arrow in A). Lying directly adjacent to the afferent and efferent arterioles and adjacent to some extraglomerular mesangial cells at the vascular pole of the renal corpuscle is the terminal portion of the distal straight tubule of the nephron. At this site, the wall of the tubule contains cells that are referred to collectively as the macula densa (rectangles in B). DT – Distal tubules; PT – Proximal tubules.

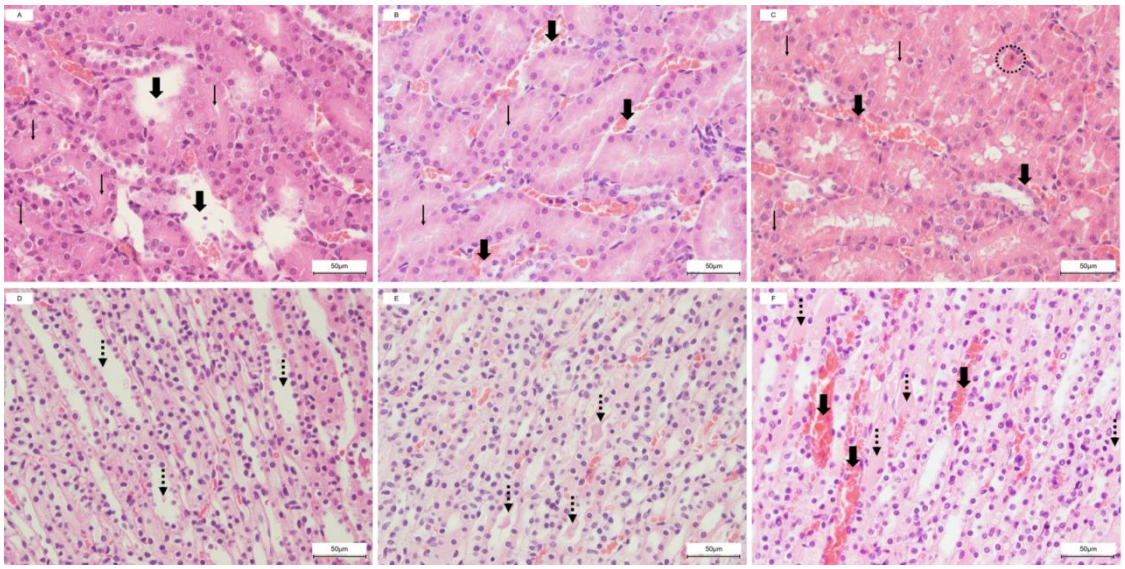

Figure 16. Representative liver images from rat treated with LAS (A and D), with OR (B and E) and with OR+LAS (C and F). In kidney cortex proximal tubules (thin arrows in A, B and C) presented structural changes include those of reversible injury such as cellular swelling, loss of brush border, blebbing, and cell detachment and those associated with lethal injury necrosis and apoptosis (cell surrounded by the circle in C). In kidney medulla collecting ducts contain hyaline casts (dashed arrow). In entire organ area it is possible to observe sinusoidal dilatation (thick arrows).

4.1.5.2 Liver

The rat liver of the control group exhibited the morphological features described for this species with no relevant histological alteration (Figure 17). Necrotic areas with clustered of affected hepatocytes and inflammatory infiltrate were identified in rat livers of all experimental groups, LAS, OR and OR+LAS sorted this way in ascending order of

apparent volume of the lesions. Liver biopsy showed mild lymphocytic portal inflammation, the bile ducts were intact and there was no ductular reaction. Foci of lobular inflammation were present (Figure 18A, herein illustrated for LAS group). Small foci of lobular inflammation were also present in all experimental groups (Figure 18B, herein illustrated for OR group).

Confluent perivenular submassive necrosis of liver calls was present, with extension to the midzones and periportal zones, and with associated haemorrhage within the liver cell plates (Figure 18C and D, herein illustrated for LAS and OR groups, respectively).

The necrotic cells showed increased cytoplasm eosinophilia and a small, basophilic nucleus. Rats exposed to OR+LAS had livers with an apparent higher volume of lesions and with two specific ones. It was found a severe sinusoidal dilatation and haemorrhage (Figure 18E) and near vessel walls hepatocytes with hydropic degeneration were observed (Figure 18F).

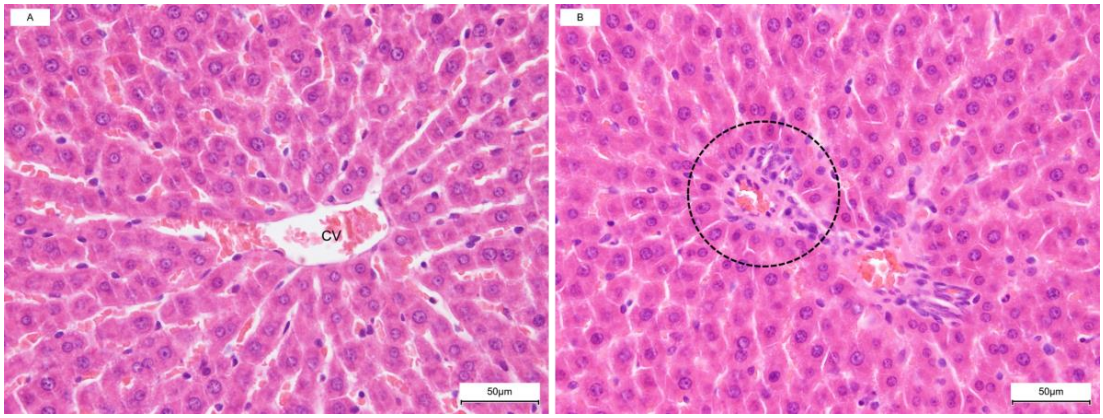

Figure 17. Representative images from rat of the control group. (A) In this photomicrograph the terminal hepatic venule (central vein, CV) is visible in the centre of the lobule. The hepatocytes are acidophilic and form plates and are in close contact with the sinusoids. (B) In these image marked with the circle is a portal triad.

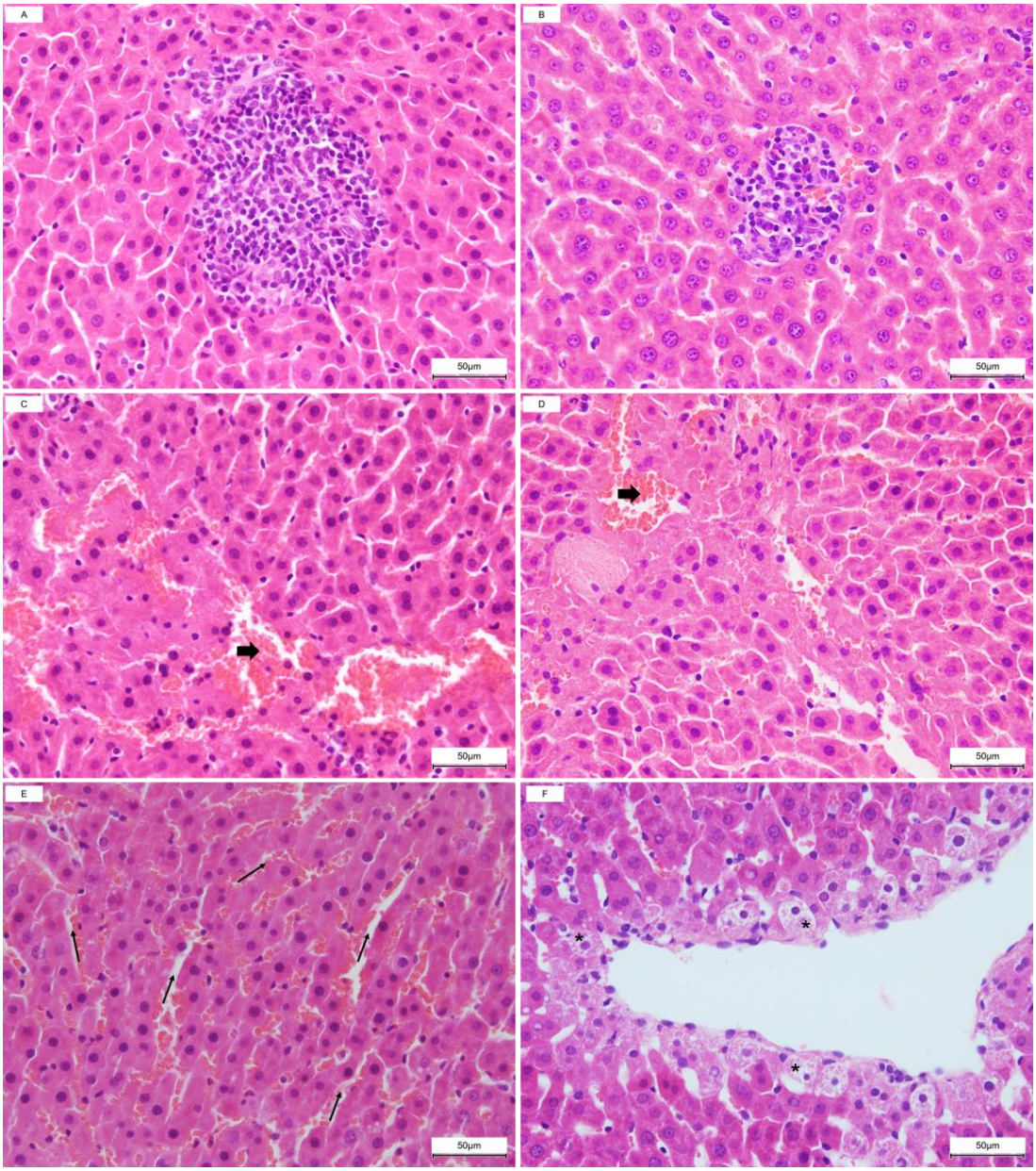

Figure 18. (A) Representative liver image from rat treated with LAS. The portal tract exhibits a moderate lymphocytic infiltrate. (B) Representative liver image from rat treated with OR showing mild lobular inflammatory activity. (C) Representative liver image from rat treated with LAS with hemorrhagic necrosis (arrow) (D) Representative liver image from rat treated with OR where parenchymal damage, hemorrhage, and absence of viable hepatocytes were evident. (E) Representative liver image from rat treated with OR+LAS with sinusoidal dilatation (arrows). (F) Representative liver image from rat treated with OR+LAS where is possible to observe a hydropic change of hepatocytes (*) near to the wall of the vessel.

4.1.5.3 Lung

In the lungs of the rats of control group alveoli, the terminal air spaces of the respiratory system and the sites of gas exchange between the air and the blood, were perfectly normal (Figure 19A). In animals treated with LAS (Figure 19B) and with OR (Figure 19C), the alveolar capillaries were distended. Animals treated with OR+LAS (Figure 19D) exhibited an enlargement of alveoli accompanied by destruction of their walls without obvious fibrosis.

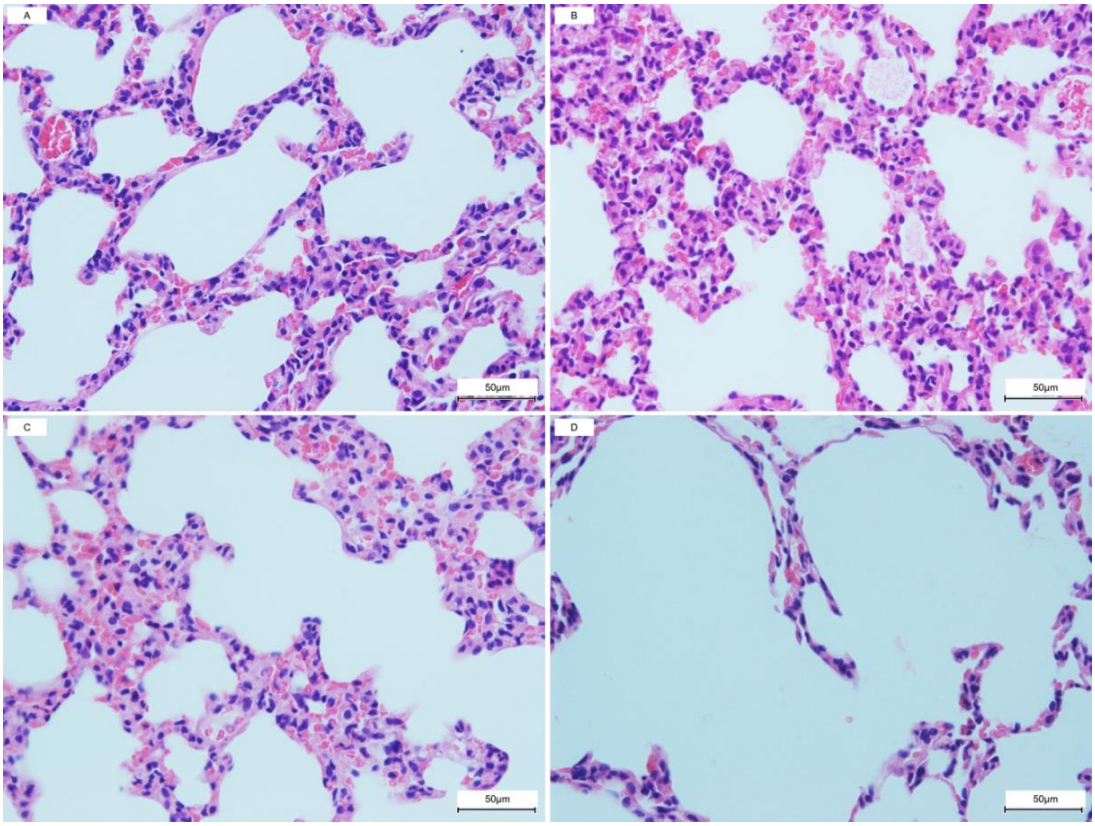

Figure 19. Representative lung images from control- (A), LAS- (B), OR- (C) and OR+LAS-treated rats (D). Dilatation of alveolar capillaries can be observed in lungs of animals treated with LAS (B) and with OR (C). Enlargement of alveoli accompanied by destruction of their walls without obvious fibrosis was observed in animals treated with OR+LAS (D).

4.1.5.4 Spleen

Compared with the spleen of the control animals, that presented the microanatomy described for the species (Figure 20), the spleen from an OR-treated mouse revealed an overall reduction in red and white pulp cellularity (Figure 21). Microscopically, the major feature of the spleen of the animals treated with OR and with OR+LAS was an acute congestion of the red pulp, which may invade on and virtually efface the lymphoid follicles.

The acute congestion is in line with a clear increase of red cells volume. Neutrophils, plasma cells, and occasionally eosinophils were also present throughout the white and red pulp.

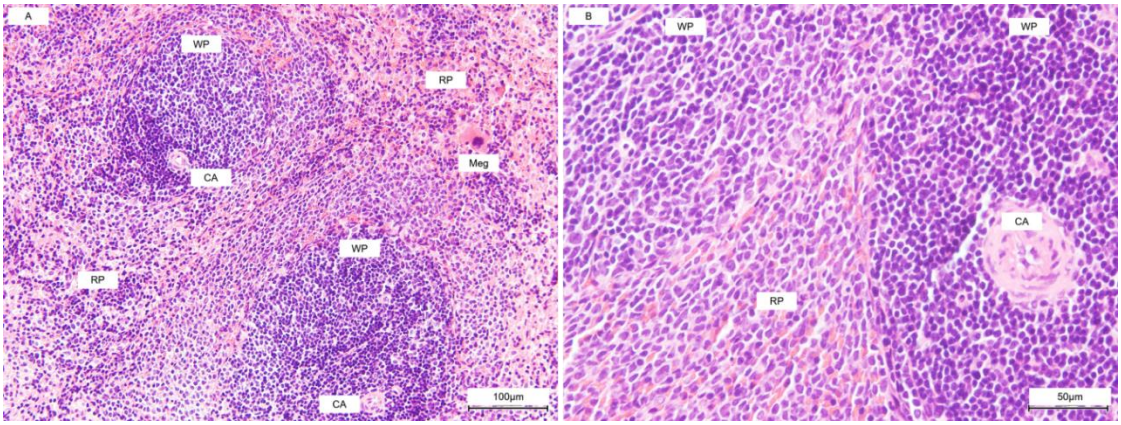

Figure 20. Representative images from rat spleen of the control group. The substance of the spleen is divided into white pulp (WP) and red pulp (RP). White pulp consists of a cylindrical mass of lymphocytes arranged around a central artery (CA) that constitutes the periarterial lymphatic sheath. The red pulp consists of splenic sinuses surrounded by splenic cords (cords of Billroth). In rat, the splenic red pulp is a major site of myeloid, erythroid hyperplasia and megakaryocytic hyperplasia (Meg - megakaryocyte).

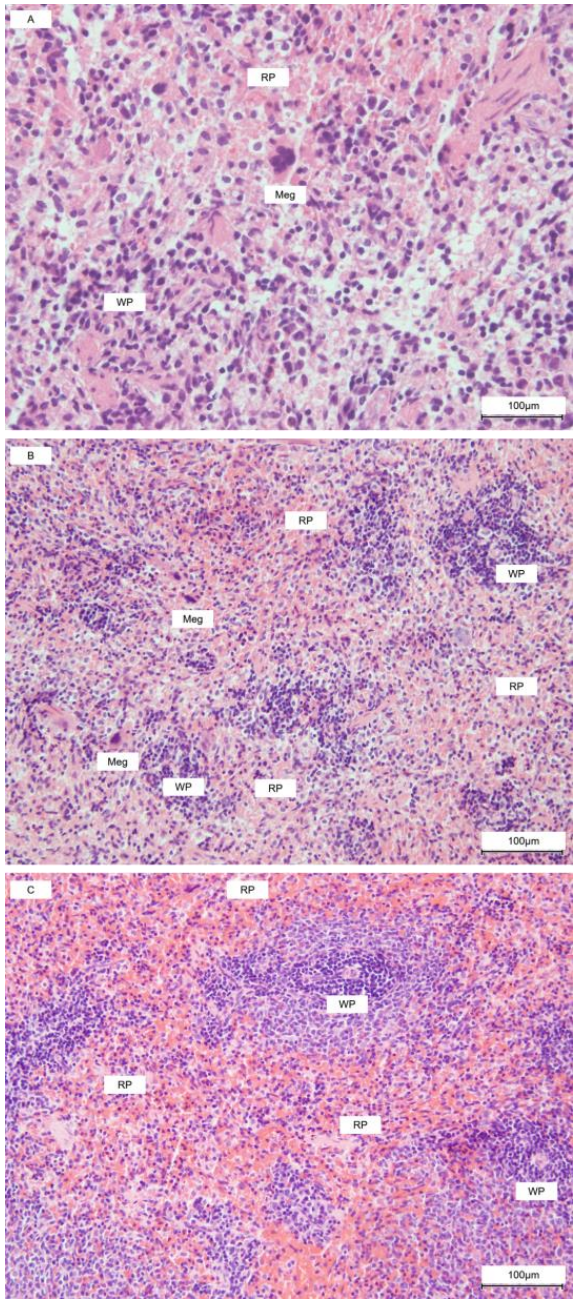

Figure 21. Representative spleen images from rat treated with LAS (A), OR (B) and OR+LAS (C). In all images it is possible to observe an overall reduction in red and white pulp cellularity and also a congestion of the red pulp due to a clear increase of red cells volume especially in animals treated with OR+LAS.

4.1.5.5 Heart

The cardiac muscle of the rats of both control and LAS groups revealed the histological features described for this species. In longitudinal sections the cells and the fibres they form exhibit cross-striations that were evident. In addition, it was also possible

to observe the intercalated discs in the cardiac muscle fibres (data not shown). In the rats treated with OR and with OR+LAS, circumscribed areas of ischemic necrosis - coagulative necrosis of myocardial fibres were well delineated. These muscle cells presented intense eosinophilic cytoplasm, features typical of a very recent myocardial infarct (Figure 22).

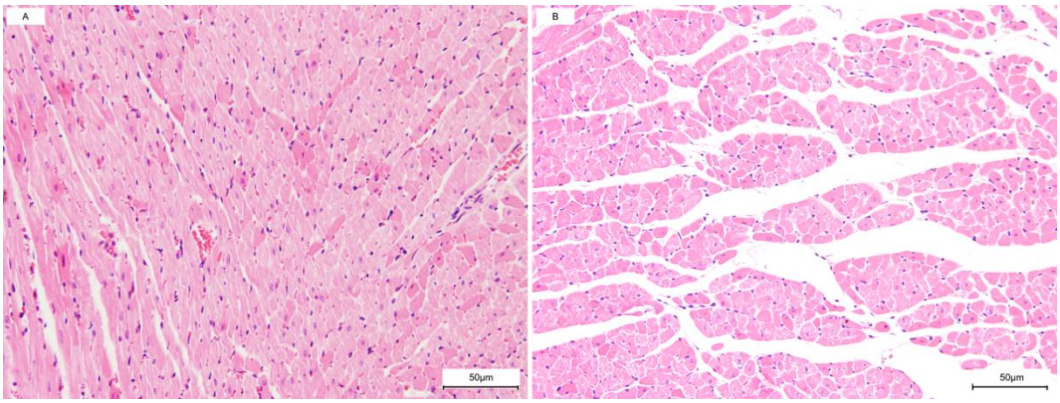

Figure 22. Representative heart images from rat treated with OR (A) and OR+LAS (B). In both images is possible to observe a mosaic of cells composed by normal and necrotic myocardial fibers, the latter with intense eosinophilic cytoplasm.

4.1.6 Measurement of total RNA

OR inhibits DNA-depended activity of RNAP II with a consequent decrease on messenger RNA (mRNA). We quantified the total RNA content in the kidney, liver, lung, spleen and heart of animals in all experimental groups. These total RNA levels were further normalized to organ weight. However, no differences were found for total RNA in the kidney, lung, spleen and heart of OR-intoxicated animals (Table 8). It was observed only a tendency to decreased total RNA liver levels in the OR group (1.35±0.44 µg total RNA/mg) when compared to control group (2.87±0.53 µg total RNA/mg) (Figure 24).

Table 8 – Total RNA kidney, liver, lung, spleen and heart levels of control, OR, LAS and OR+LAS groups.

	Control	OR	LAS	OR + LAS
Kidney	1.00±0.21	1.20±0.15	1.15±0.40	0.73±0.32
Liver	2.87±0.53	1.35±0.44	1.88±0.48	2.07±0.66
Lung	0.97±0.26	0.89±0.23	0.58±0.08	0.88±0.20
Spleen	1.34±0.65	0.78±0.10	2.06±0.77	2.13±0.52
Heart	0.89±0.10	1.22±0.25	0.79±0.22	0.63±0.13

Values are given as mean±SEM (*n* = 5)

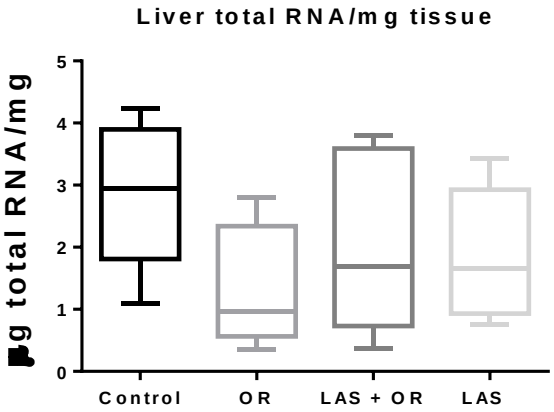

Figure 23. (A) Total RNA liver levels in control, OR, LAS and OR+LAS groups. Results are presented as mean ± SEM, and were obtained from 5 animals from each treatment. Statistical comparision were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test*.

4.1.7 Alterations of the transcription process

The evaluation of the genetic transcription by RNAP II was based on GAPDH and β-actin mRNA quantitative analysis. The relative transcript levels were quantified by the Ct value, which increases with a decreasing amount of template.

The transcript levels of GAPDH mRNA in the liver samples of OR-treated showed a decreased (20.66±0.38, p<0,05) when compared with control group (18.06±0.66). This effect was reverted in the OR+LAS group (17.88±0.37, p<0,01) (Figure 24 A). Data from OR-intoxicated liver indicated that the transcription of β-actin mRNA showed a tendency to decrease (25.01±0.77), but no statistically significance (Figure 24 B) (Table 9). No significant differences were observed between OR group and the control group concerning the transcript levels of GAPDH and β-actin mRNA in the kidney, lung, spleen and heart (Table 9).

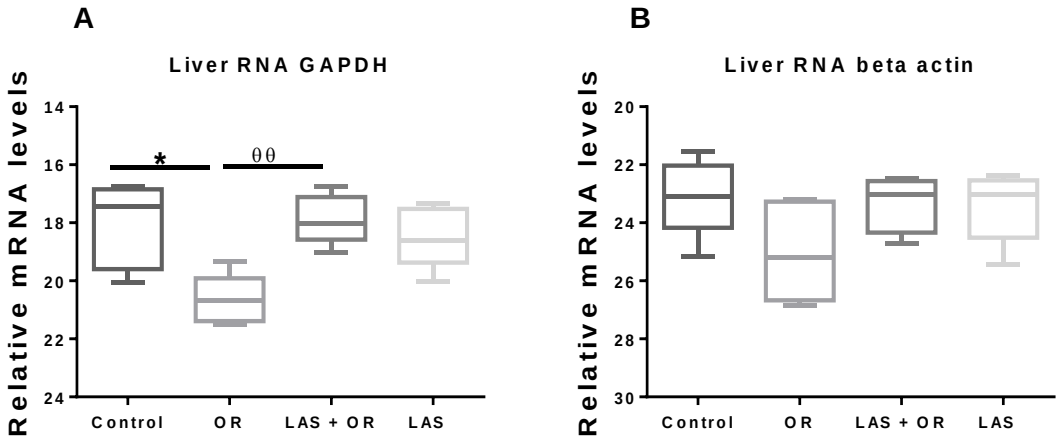

Figure 24. Relative mRNA levels of GADPH and β-actin genes in liver samples. Statistical comparision were using Kruskal-Wallis ANOVA on Ranks followed by the Dunn's *post hoc test* (*p < 0.05 OR vs. Control; $^{\theta\theta}$ *p* < 0.01 OR *vs* OR+LAS).

Also be added, RNAP I was not affected by OR poisoning since the transcription of ribosomal proteins S18 and S28 by RNAP I was always similar, regardless of the organ or experimental groups analyzed (Table 9).

Table 9 – Relative mRNA levels of GADPH, β-actin, S18 and S28 genes in kidney, liver, lung, spleen and heart samples.

	RNAP II		RNAP I	
	GADPH	**β-actin**	**S18**	**S28**
Kidney:				
Control	19.29±0.71	22.77±1.60	18.45±0.84	23.12±0.96
OR	17.78±0.70	22.62±0.78	17.70±0.57	21.69±0.58
LAS	19.16±0.90	23.18±1.36	18.55±0.76	23.15±1.08
OR + LAS	20.67±1.45	23.28±0.78	20.07±1.60	22.59±0.57
Liver:				
Control	18.06±0.66	23.09±0.59	18.84±0.80	22.46±0.70
OR	20.66±0.38*	25.01±0.77	18.75±0.59	22.67±0.34
LAS	18.48±0.47	23.42±0.54	18.21±0.65	22.93±0.61
OR + LAS	17.88±0.37$^{\theta\theta}$	23.36±0.42	17.92±0.33	21.91±0.36
Lung:				
Control	20.63±1.20	19.41±1.58	21.81±1.75	21.53±0.44
OR	18.69±1.20	22.89±1.26	18.68±1.72	21.96±0.73
LAS	19.57±0.96	23.12±1.041	18.91±1.54	22.38±0.56
OR + LAS	19.50±1.32	19.91±1.28	20.12±1.25	22.76±0.76
Spleen:				
Control	18.46±1.25	21.36±1.68	18.50±0.65	21.30±1.12
OR	18.32±1.33	21.89±1.47	17.69±1.43	21.65±1.48
LAS	18.74±0.67	20.30±1.33	17.21±1.20	20.32±0.73
OR + LAS	19.00±1.22	19.47±0.86	17.65±0.60	20.80±0.78

Heart:				
Control	20.29±1.08	24.57±1.32	20.90±1.08	21.18±0.96
OR	19.02±0.50	22.98±0.55	19.31±0.89	22.81±0.21
LAS	18.58±0.75	24.00±0.62	18.24±0.68	22.73±0.62
OR + LAS	19.50±0.51	24.80±0.90	19.50±0.34	23.56±0.76

Values are given as mean±SEM (n = 5); * $p < 0.05$ OR *vs* Control; $^{\theta\theta}$ $p < 0.01$ OR *vs* OR+LAS.

4.2 Survival rate study

A long-term survival study for 5 months was done. All mice exposed to 3.5 mg/kg of orellanine did not die. The administration of OR+LAS resulted in 100% of survival.

Part V

Discussion

5. DISCUSSION

In this dissertation two types of *in vivo* studies were performed, in order to prove the effectiveness of LAS against OR intoxication: it was executed a short-term study (sacrifice time at 24 hours) and a survival study (5 months).

Since kidney injury is the main concern in OR poisoning, kidney damage biomarkers were assessed, namely serum creatinine, total protein, uric acid and urea. Laboratory abnormalities by OR poisoning include leucocyturia, hematuria and proteinuria along with elevation of the serum creatinine and serum potassium. Depending on the severity of the renal failure, hyperphosphatemia, hypocalcemia and metabolic acidosis may also occur (Barceloux, 2008). The increasing in urea and creatinine levels in blood were also reported on these poisonings (Michelot and Tebbett, 1990). In other study, the levels of urea nitrogen (BUN), ALT and alkaline phosphatase (ALP) on blood were increased in a mouse 96 hours post exposed with OR (Anantharam et al., 2016). (Prast and Pfaller, 1988) have reported progressive elevation on the serum concentrations of BUN and creatinine (Prast and Pfaller, 1988). Hypocalcemia was also observed in other *in vivo* study and reduced sodium ion concentration and elevated phosphorus concentration in serum was reported (Anantharam et al., 2016). Bedry *et al.* observed a massive rhabdomyolysis and increased CK level (Bedry et al., 1998). Our results demonstrate that there was a statistically significant increase in serum HDL, uric acid and hypocalcemia. The increased UA in OR group can either be related to a possible kidney damage or to an increased catabolism of purines (Prakash, 2014). Serum levels of HDL in OR group do not produce untoward clinical manifestations and are associated with below-average risk of atherosclerosis (SarDesai, 2011). The kidney of rat administered with OR evidenced notorious damage, with tubular ectasia, tubular proteinosis, and epithelial cells of the affected tubules exhibited marked cytoplasmic degenerative changes or necrosis and apoptosis. These histological phenotypes are in agreement with previous reports of OR studies in mice (Anantharam et al., 2016), rats (Prast and Pfaller, 1988; Richard et al., 1988) and other animals (Grzymala, 1957; 1962; 1965b). Moreover, histopathological kidney damage in laboratory animals is similar to those found in humans after OR intoxication (Horn et al., 1997; Marichal et al., 1977; Rapior et al., 1989; Short et al., 1980). These histological kidney findings also show that LAS did not reverte the damage caused by OR, being that, the volume of the affected tubules was apparently higher in the rats of OR+LAS group. The proteinuria observed in these animals is likely of tubular origin

because histopathology showed tubular necrosis and, further, the increased levels of the lysossomal enzyme NAG as tubular nephrotoxicity marker. In our experimental study it was observed an increase of the pancreatic enzyme, amylase, in the group OR+LAS. The serum amylase can also be elevated in renal failure with a very low creatinine clearance (Chawla, 2014). The hypocalcemia observed in these animals may be associated with an attack of severe pancreatitis or renal failure (Chawla, 2014).

Regarding the liver, it is also an organ affected by OR poisoning. Flammer (1980) interpreted OR intoxication to consist of hepatorenal syndrome (Flammer, 1980). Grzymala (1957) diagnosed jaundice combined with liver swelling and severe liver cell necrosis as the primary lesion in the course of intoxication. Autopsy showed liver necrosis and interstitial nephritis (Grzymala, 1957). Favre et al. (1976) reported hepatocellular damage in humans on the basis of elevated activity of serum glutamatepyruvate-transaminase (SGPT) and serum glutamate-oxaloacetate-transaminase (SGOT), as well as increased plasma bilirubin (Favre et al., 1976). In our study, mild lobular inflammatory activity, parenchymal damage, hemorrhage and absence of viable hepatocytes were evident in animals treated with OR. In other study, these findings, mainly minor hepatocellular vacuolation, was reported (Anantharam et al., 2016). In some animals, foci of necrotic cells in the liver were also reported (Schumacher and Hoiland, 1983), as well as liver enlargement, fatty degeneration and some necrotic patches in liver cells in intoxicated people (Gryzmala, 1965; Grzymala, 1959b). The absence of a LAS-mediated protective effect was again observed. Consequently, the animals exposed to OR+LAS had livers with an apparent higher volume of lesions.

In this study, the CK levels were significantly increased in the OR+LAS group, which suggests muscle damage. Clinically, CK is used as a marker of myocardial infarction (heart attack), rhabdomyolysis (severe muscle breakdown), muscular dystrophy, autoimmune myositides and acute renal failure (Chawla, 2014). Hypoalbuminemia is common in patients with heart failure (Arques and Ambrosi, 2011). In our study, it was observed a decreased albumin in the heart of animals treated with OR+LAS. That being said, the serum findings were corroborated by histological observations. It was observed, both in the OR and OR+LAS groups, circumscribed areas of ischemic necrosis. A novel histopathological finding shown in the present study was the myocardial infarct.

Dilatation of alveolar capillaries can be observed in lungs of animals treated with OR. Lungs of rats administered with OR+LAS exhibited an alveoli accompanied by destruction of their walls without obvious fibrosis. This experimental evidence suggests that the administration of LAS may aggravate the pulmonary toxicity of OR.

Another observation taken from this study was an acute congestion of the red pulp in the spleen of both OR and OR+LAS groups, characterized by an increase of red cells volume. Neutrophils, plasma cells, and occasionally eosinophils were also present throughout the white and red pulps. In both cases, the toxicity induced for OR was not reversed with the administration of LAS. Anantharam *et al.* reported splenic atrophy, characterized by lymphocytolysis in OR-treated mice. The few studies involving rats have been focusing on the renal effects of *Cortinarius* mushrooms ingestion. Other organs, such as the lungs, heart and spleen, were not examined histologically (Anantharam et al., 2016).

In this study, we could not observe differences on organ to body weight ratio between control and treated groups. In other study, intoxicated rats with OR showed a significantly greater loss of body weight (Prast and Pfaller, 1988). Anantharam *et al.* reported a statistically significant reduction in organs weight, namely in liver and spleen of mice given OR when compared to control group (Anantharam et al., 2016)

Glutathione is an important antioxidant that plays a major role in protecting cells against oxidative stress (Townsend et al., 2003) . GSH depletion may render the cells more vulnerable to the deleterious effects of free radicals, increasing their susceptibility towards oxidative damage. The cellular balance of GSH and GSSG provides a dynamic indicator of oxidative stress. That is, during acute oxidative stress, it is observed a decrease in GSH concentration with an associated increase in GSSG concentration (Jones, 2002). The most widely used indicator of the redox environment of the cell is the ratio of GSH to GSSG (Jones, 2002; Valko et al., 2007). Oxidative stress has also been implicated in OR-induced kidney injury (Cantin et al., 1988; Nilsson et al., 2008; Oubrahim et al., 1998; Richard et al., 1995). Prast reported, on kidneys exposed to OR, peroxidative cell damage, namely the reduced tissue content of glutathione (Prast, 1982). Nilsson *et al.* studied the renal mRNA levels in rats exposed to OR for a set of genes with relevance to oxidative stress and antioxidant defense. These investigators reported a strongly decrease of the antioxidant defense, namely catalase (CAT), cytoplasmic CuZn-SOD (SOD1), and plasma glutathione peroxidase (GPX3), and a significant increase of several inflammatory cytokines (IL1b, TGF1β, TNF) (Nilsson et al., 2008). The oxidative stress biomarker, GSSG, was markedly increased in the spleens of OR group. As a consequence, the ratio GSH/GSSG was decreased. The kidney and liver GSSG levels were significantly increased in OR+LAS group. In accordance, the GSH/GSSG ratio suffered a significant reduction. In this study, a tendency to an increase in lipid peroxidation in spleen, heart and lungs was observed in animals treated with OR+LAS, but it did not reach statistical significance. The amount of MDA in kidney was slightly large

in OR group but not statistically significant. In other study, OR applied *in vitro* to kidney homogenate resulted in an enhanced formation of thiobarbituric acid reactive substances (largely malondialdeyde) (Prast and Pfaller, 1988). These results confirm that LAS does not confer protection against OR-induced oxidative stress.

In the literature, OR inhibits DNA-depended activity of RNAP II, therefore, the RNA levels was used as a measure of its inhibition. Our results showed an inhibition of liver GAPDH mRNA transcription elicited by OR. Other study, showed that *C. orellanus* toxin inhibits DNA-depended activity of RNA polymerase II from rat liver and DNA-depended activity of RNA polymerase from Escherichia coli (Moser, 1969).

The present study provides *in vivo* evidence that LAS does not confer a protection against OR induced-toxicity. Indeed, LAS treatment clearly increased the toxicity of OR. It is necessary to understand what mechanism is underlying the increased toxic action of OR associated with LAS. Experimental study with rats suggested that high-dosed LAS increases the risk of toxicity and, in lower doses, the protective effect might be insufficient. Dinis-Oliveira and co-authors demonstrated that a LAS dose of 200 mg/kgallowed the full survival of PQ+LAS-treated rats, but doses of 100 and 400 mg/Kg only allowed 60% and 20% of survival, respectively (Dinis-Oliveira et al., 2009a). According to the literature, the toxicity in adults or children attributable to high doses of salicylate results in various clinical manifestations depending on plasma concentrations. Mild toxicity occurs for 300–500 mg/L, moderate toxicity occurs for 500–700 mg/L and severe cases ensue for plasma concentrations higher than 750 mg/L (Done, 1960; Proudfoot, 1983). In mild or early poisoning, burning in the mouth, lethargy, nausea, vomiting, tinnitus, or dizziness can occur. In moderate poisoning all of the above plus tachypnea, hyperpyrexia, sweating, dehydration, loss of coordination, and restlessness, can occur (Prescott et al., 1982; Proudfoot, 1983). In severe poisonings, hallucinations, stupor, convulsions, cerebral edema, oliguria, renal failure, cardiovascular failure, and coma may be seen together with metabolic acidosis (Chapman and Proudfoot, 1989; Proudfoot, 1983; Thisted et al., 1987).

Other studies, to reverse the renal damage induced of OR were performed but without effect. Nieminen *et al.* studied the effect of phenobarbital and phenylbutazone treatment on the renal damage induced by the toxic mushroom *C.speciosissimus* in female rats. The effect on renal toxicity in rats by the mushroom extract was investigated after 11 days of pretreatment with phenobarbital. It was found that the phenobarbital treatment strongly increased the damage induced by *C. speciosissimus* in the tubules of renal cortex but had no effect on the inflammation in the outer medullary zone induced by this toxic mushroom. Phenylbutazone treatment had no effect on the renal damage induced by these mushroom (Nieminen et al., 1976). The same group, also studied the effect of furosemide

on the renal damage induce by the toxic mushroom *C. speciosissimus* in female rats. It was observed that coadministration of furosemide caused marred necrotic changes in the tubules of the outer cortical layer, whereas in animals receiving only the mushrooms the outer cortex was clear. On the other hand, furosemide had no effect on the degree of inflammation. Furosemide clearly increased the nephrotoxicity of *C. specisossimus* (Nieminen et al., 1976).In other study, Nilsson and collaborators studied the effect of superoxide dismutase (SOD) on the kidney damage induced by the OR in female rats. The treatment with SOD during the 4 days after OR injection led to even further decreased renal function. This treatment also increased mortality within the first week after OR injection from 0% in the OR group to 33% in the OR + SOD group. This was reflected also in the amount of oxidative protein damage in the tubular epithelium, which was elevated, compared to the control group, in the OR group and even higher in the OR + SOD group (Nilsson et al., 2008).

The hindrance of OR toxicity by LAS was established by a 5 months survival study. The administration of LAS at 4 h post OR and OR-treated animals resulted in a 100% of survival rate. The data demonstrate that the OR dose was not sufficient to cause death. In this experimental approach, LAS was administered 4 h after orellanine exposure, seeking a more realistic treatment approach, since hospitalization after *Cortinarius* poisoning usually occurs only hours and days after ingestion.

Part VI

Conclusions and Future Perspectives

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, the dosage regimen of LAS used did not promoted protection on oxidative stress parameters in the organs of animals treated with OR. There were some organs where damage could be observed, namely in kidney, liver, lung, heart and spleen. However, with this study, it was given an important step in knowledge on OR-induced pathology. Cardiac, splenic and lung injuries are novel findings in rats intoxicated with OR.

The mechanism for the increase of toxicity by the coadministration of LAS needs further studies. It is necessary to test lower doses of LAS, seeing that the dose utilized demonstrated to be excessive. Additionally, it is also necessary to test higher doses of OR for the purpose of finding the effective dose LD50 in mice, since with our dose all animals survived.

Concerning future perspectives, additional knowledge is necessary to evaluate a long term toxicity of OR and the biochemical effects, to take measures suitable to avoid fatal consequences, by developing effective therapies to counteract toxic effects. Indeed, it would be interesting to find out what degree of kidney, heart, liver, lung and spleen damage occurs 14 days after the administration of the OR (latent period). Further knowledge of OR toxicokinetics is also necessary.

Part VII

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ATTACHEMENTS

A1 – Kidney biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	1.09±0.11	0.75±0.18	0.90±0.063	0.81±0.15
TP (g/L)	23.05±*1.43*	23.71±*4.50*	18.18±*0.86*	16.97±*2.91*
Chol (mg/dL)	43.02±*4.92*	64.00±*27.89*	54.86±*11.21*	26.66±*6.28*
HDL (mg/dL)	6.08±*1.80*	9.76±*4.29*	8.00 ±*1.96*	6.68±*1.41*
LDL (mg/dL)	58.92±*8.44*	61.12±*15.72*	86.30±*11.96*	50.98±*6.75*
Glucose (mg/dL)	23.96±*4.25*	18.38±*5.69*	21.80±*.929.00*	17.36±*2.91*
Creatinine (mg/dL)	0.08±*0.04*	0.02±*0.02*	0.17 ±*0.07*	0.12±*0.07*
UA (mg/dL)	3.58±1.21	2.48±0.97	2.96±1.28	2.30±1.11
TG (mg/dL)	155.60±58.78	63.75±13.29	101.60±26.84	123.20±33.77
Amylase (U/L)	118.90±28.57	116.00±2332.00	72.50±14.25	50.42±10.37
ALAT (U/L)	0.70±0.20	2.36±1.00	1.58±1.48	0.90±0.56
ASAT (U/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
LDH (U/L)	15.68±3.77	10.75±6.47	14.60±2.97	15.18±3.71
CK (U/L)	800.70±239.90	1386.00±386.20	1059.00±207.80	1369.00±351.90
CKMB (U/L)	1372.00±392.00	1465.00±413.40	835.90±290.80	953.10±372.90
Chloride (mg/dL)	174.10±66.14	50.93±12.11	187.60±32.43	92.90±25.39
Phos (mg/dL)	9.81±1.82	15.34±3.03	9.30±1.29	16.67±3.10
Mg (mg/dL)	3.58±0.22	3.52±0.26	3.44±0.26	3.91±0.43
Ca (mg/dL)	1.03±0.10	1.10±0.040	1.30±0.34	0.80±0.11
Iron (mg/dL)	1140.00±503.70	517.10±103.20	863.40±219.50	604.00±234.60
GGT (U/L)	0.00±0.00	30.76±21.21	0.00±0.00	0.00±0.00
Lactate (mg/dL)	19.80±1.02	13.80±3.96	29.40±3.71	23.80±1.99

Values are given as mean±SEM (*n* = 5).

A2 – Liver biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	2.92±0.24	2.95±0.14	3.15±0.06	3.02±0.134
TP (g/L)	137.30±6.14	140.00±11.10	132.50±6.20	128.40±7.985
Chol (mg/dL)	356.50±26.59	363.50±43.23	365.50±22.36	339.60±15.09
HDL (mg/dL)	66.96±7.59	68.16±6.92	68.12±20.37	50.62±13.74
LDL (mg/dL)	479.90±54.77	491.40±63.11	462.90±48.70	452.40±48.1
Glucose (mg/dL)	180.20±19.15	196.00±15.96	189.70±19.78	256.10±33.19
Creatinine (mg/dL)	0.47±0.29	0.20±0.14	0.36±0.23	0.60±0.2146
UA (mg/dL)	13.30±3.15	12.98±5.20	13.98±4.16	14.14±5.773
TG (mg/dL)	515.00±104.10	480.20±85.02	488.70±161.90	425.90±27.09
Amylase (U/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
ALAT (U/L)	0.92±0.023	0.90±0.00	0.90±0.00	0.90±0.00
ASAT (U/L)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
LDH (U/L)	15.68±3.77	10.75±6.47	14.60±2.97	15.18±3.71
CK (U/L)	83.28±20.53	51.44±16.48	55.32±24.87	32.60±10.23
CKMB (U/L)	0.00±0.00	16.71±10.99	0.00±0.00	54.98±38.06
Chloride (mg/dL)	94.68±35.00	71.98±11.73	143.10±27.93	159.30±53.31
Phos (mg/dL)	0.00±0.00	10.99±6.77	0.00±0.00	0.00±0.00
Mg (mg/dL)	8.46±0.13	8.28±0.49	7.69±0.22	7.93±0.44
Ca (mg/dL)	1.86±0.32	2.10±0.24	1.73±0.42	1.78±0.19
Iron (mg/dL)	3740.00±167.90	5163.00±491	5410.00±646.90	5432.00±497.00
GGT (U/L)	31.26±5.52	18.32±9.41	32.96±7.07	11.34±3.07
Lactate (mg/dL)	91.40±6.64	107.20±15.67	92.20±6.40	106.60±11.01

Values are given as mean±SEM (*n* = 5).

A3 – Lung biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	0.22±0.09	0.42±0.06	0.13±0.09	0.12±0.07
TP (g/L)	29.24±*6.92*	13.68±*1.32*	11.89±*2.36*	38.11±*13.43*
Chol (mg/dL)	73.24±*40.35*	21.08±*3.12*	22.84±*5.90*	20.23±*6.77*
HDL (mg/dL)	0.80±*0.34*	1.48±0.35	1.80±*0.64*	3.12±0.77[#]
LDL (mg/dL)	26.12±*2.74*	29.66±*3.167*	22.92±*7.01*	28.16±*5.61*
Glucose (mg/dL)	22.14±*12.63*	7.64±*1.22*	6.42±*0.93*	11.28±*3.27*
Creatinine (mg/dL)	0.08±*0.05*	2.71±*1.75*	0.13 ±*0.01*	0.01±*0.01*
UA (mg/dL)	4.46±0.98	4.42±0.27	3.10±0.33	2.92±0.58
TG (mg/dL)	110.60±36.36	105.00±20.53	29.96±8.87	74.98±23.33
Amylase (U/L)	23.58±1.53	13.05±4.85	18.16±7.50	13.60±2.48
ALAT (U/L)	1.45 ±0.89	3.60±0.94	2.90±0.40	1.65±0.79
ASAT (U/L)	0.76±0.49	0.00±0.00	0.08±0.08	0.00±0.00
LDH (U/L)	534.60±182.50	388.90±81.93	783.60±174.70	5778.20±180.50
CK (U/L)	526.10±228.80	1020.00±466.60	1985.00±388.60	1670.00±484.80
CKMB (U/L)	1807.00±288.80	1776.00±432.70	1503.00±400.10	1589.00±177.60
Chloride (mg/dL)	28.33±4.20	65.90±25.39	28.36±11.09	64.76±27.37
Phos (mg/dL)	29.40±1.24	31.56±0.30	32.03±0.16	31.34±0.42
Mg (mg/dL)	2.46±0.32	2.29±0.51	2.59±0.42	3.33±0.67
Ca (mg/dL)	0.83±0.21	1.33±0.46	0.94±0.24	0.68±0.20
Iron (mg/dL)	399.70±223.90	442.30±252.40	156.80±47.48	145.80±25.84
GGT (U/L)	0.00±0.00	3.65±3.65	1.80±1.25	1.08±1.08
Lactate (mg/dL)	13.40±0.40	12.00±0.84	10.80±0.37	10.80±1.11

Values are given as mean±SEM (*n* = 5); * $p < 0.05$ OR *vs*. Control; # $p < 0.05$ OR+LAS *vs*. Control; $^{\theta}$ $p < 0.05$ OR *vs* OR+LAS.

A4 – Spleen biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	0.14 ±0.05	0.126±0.02821	0.00±0.00	0.03±0.02
TP (g/L)	7.39±*0.88*	7.145±*0.3835*	7.70±*0.65*	6.03±*0.96*
Chol (mg/dL)	10.70±*2.15*	9.9±*1.641*	18.18±*3.60*	17.04±*8.01*
HDL (mg/dL)	1.48±*0.30*	2.22±*0.4247*	1.92±*0.65*	3.32±*1.51*
LDL (mg/dL)	9.76±*3.21*	10±*1.39*	9.86±*2.98*	13.66±*4.25*
Glucose (mg/dL)	2.52±*1.39*	0.46±*0.2205*	3.64±*0.99*	3.90±*1.80*
Creatinine (mg/dL)	0.01±*0.005*	0.0125±*0.0075*	0.03 ±*0.026*	0.05±*0.02*
UA (mg/dL)	1.86±0.82	1.08±0.3929	1.26±0.45	0.94±0.31
TG (mg/dL)	16.04±6.97	15.04±7.043	25.98±7.64	8.58±4.01
Amylase (U/L)	122.90±77.36	16.65±12.08	18.40±8.47	11.95±6.08
ALAT (U/L)	2.30 ±0.43	3.04±1.189	2.78±0.90	2.98±0.92
ASAT (U/L)	0.23±0.23	0.00±0.00	0.00±0.00	0.00±0.10
LDH (U/L)	1009.00±197.30	980.80±112.30	735.40±131.20	1083.00±182.10
CK (U/L)	2600.00±111.00	2571.00±81.91	2196.00±328.30	2184.00±293.20
CKMB (U/L)	1745.00±217.60	1975.00±171.40	1777.00±247.70	1545.00±301.70
Chloride (mg/dL)	10.90±3.81	3.6.00±1.217.00	17.64±8.82	5.45±1.04
Phos (mg/dL)	31.66±0.39	32.04±0.28	31.59±0.64	31.41±0.87
Mg (mg/dL)	2.72±0.53	3.61±0.70	3.17±0.69	2.53±0.63
Ca (mg/dL)	1.22±0.21	1.71±0.35	0.72±0.08	1.12±0.29
Iron (mg/dL)	507.40±234.40	442.70±144.50	282.80±43.13	392.80±86.44
GGT (U/L)	12.30±3.54	8.90±4.26	9.28±6.87	12.62±4.60
Lactate (mg/dL)	7.20±0.66	7.25±0.48	8.40±0.51	6.60±0.60

Values are given as mean±SEM (*n* = 5)

A5 – Heart biochemistry.

Evaluated parameter	Control	OR	LAS	OR + LAS
Albumin (g/L)	0.16±0.042	0.10±0.03	0.02±0.01	0.05±0.01
TP (g/L)	8.21±*0.95*	9.51±*1.81*	6.03±*1.464*	10.22±*2.09*
Chol (mg/dL)	8.66±*1.76*	11.78±*4.78*	6.66±*3.00*	11.26±*2.64*

HDL (mg/dL)	2.84±*0.61*	1.74±*08*	2.90±*1.67*	3.74±*0.51*
LDL (mg/dL)	9.34±*1.78*	14.00±*3.18*	4.74±*2.38*	19.18±*6.70*
Glucose (mg/dL)	8.16±*1.08*	8.66±*3.08*	5.96±*2.72*	9.22±*2.07*
Creatinine (mg/dL)	0.06±*0.04*	0.16±*0.13*	0.002±*0.0025*	0.09±*0.59*
UA (mg/dL)	1.00±0.30	0.43±0.10	1.00±0.34	0.48±0.14
TG (mg/dL)	44.28±14.15	45.60±16.43	22.72±6.44	16.80±3.47
Amylase (U/L)	19.88±5.98	8.35±2.44	4.38±2.23	4.23±1.55
ALAT (U/L)	5.24±2.53	10.42±6.59	0.35±0.21	9.70±4.41
ASAT (U/L)	1.58 ±1.58	6.36 ±3.96	0.15 ±0.15	0.00±0.00
LDH (U/L)	12.88±7.30	10.30±3.28	191.8±96.97	10.28±3.49
CK (U/L)	32.30±7.17	36.46±8.14	193.30±117.70	53.82±15.31
CKMB (U/L)	1127.00±226.90	1111.00±23	2047.00±336.60	995.00±290.60
Chloride (mg/dL)	32.88±7.01	23.78±4.74	10.60±3.41	19.60±4.00
Phos (mg/dL)	28.75±2.879.00	32.93±0.67$^{\theta}$	30.98±0.32	27.58±1.49
Mg (mg/dL)	2.92±0.42	3.34±0.79	2.73±0.82	2.90±0.67
Ca (mg/dL)	1.35±0.2489.00	1.45±0.35	0.81±0.13	0.69±0.12
Iron (mg/dL)	1057.00±511.60	440.00±51.40	204.80±5.91	1298.00±440.10
GGT (U/L)	22.50±14.19	14.54±6.31	56.56±16.81	10.78±3.19
Lactate (mg/dL)	19.80±2.35	20.80±2.71	15.40±1.36	19.20±1.91

Values are given as mean±SEM (n = 5); * $p < 0.05$ OR *vs*. Control; # $p < 0.05$ OR+LAS *vs*. Control; $^{\theta}$ $p < 0.05$ OR *vs* OR+LAS.

A6 – Paper in international journals

Unsolicited Review

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Human and experimental toxicology of orellanine

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Abstract

Orellanine is a nephrotoxic toxin produced by some mushroom species of the *Cortinarius* genus, typically found in Europe and North America. The nephrotoxicity of *Cortinarius orellanus* is well known and was first recognized in the 1950s when this mushroom was identified as the cause of a mass poisoning in Poland. Typically, onset of symptoms is delayed for 1–2 weeks after ingestion. Some patients suffer mild gastrointestinal discomfort in the latency period before developing signs of renal impairment due to severe interstitial nephritis, acute focal tubular damage, and interstitial fibrosis. There is no specific antidote to orellanine poisoning. The mainstay of treatment is the prevention of secondary complications of kidney failure, adequate dialysis and, in the case of incomplete recovery, management of chronic renal insufficiency.

In this work, we aim to review about *Cortinarius* species, including epidemiological studies, chemical structure, toxicokinetics, toxic doses, mechanisms of toxicity, diagnosis, prognosis, and treatment options.

Keywords

Cortinarius species, orellanine, redox cycling, poisoning, nephrotoxicity

Introduction

Orellanine is a highly nephrotoxic bipyridine *N*-dioxide found in various mushrooms of the *Cortinariaceae* family, including fool's webcap (*Cortinarius orellanus*) and deadly webcap (*Cortinarius rubellus* formerly named *Cortinarius speciosissimus* or *Cortinarius orellanoides*).[1,2] These two species are regarded as two of the world's most toxic mushrooms and have claimed several lives in Europe and North America after being confused for edible mushrooms such as *Cantharellus tubaeformis*, *Cantharellus cibarius*, and the hallucinogenic *Psilocybe* genus.[3–6] The nephrotoxicity of *C. orellanus* was first recognized in the 1950s when this mushroom was identified as the cause of a mass poisoning in Poland.[7–9]

The toxicity mechanism of orellanine is not yet fully understood. Chemically, orellanine resembles the bipyridyl herbicides diquat (1,1′-ethylene-2,2′-bipyridylium) and paraquat (1,1′-dimethyl-4,4′-bipyridylium; Figure 1), and some authors suggested a similar mono-electronic reduction mechanism (generation of a stable free radical) with strong prooxidant properties.[10] In accordance, experimental evidences have indicated that cytotoxic effects of orellanine are caused by the production of reactive oxygen species and subsequent oxidative stress.[11–13]

Symptoms of orellanine poisoning are those resulting from renal damage (e.g. digestive signs, thirst, headache, muscle pain, flank pain, polyuria followed by oligoanuria) developing over several days or in some cases up to 2 weeks after intoxication.[14]

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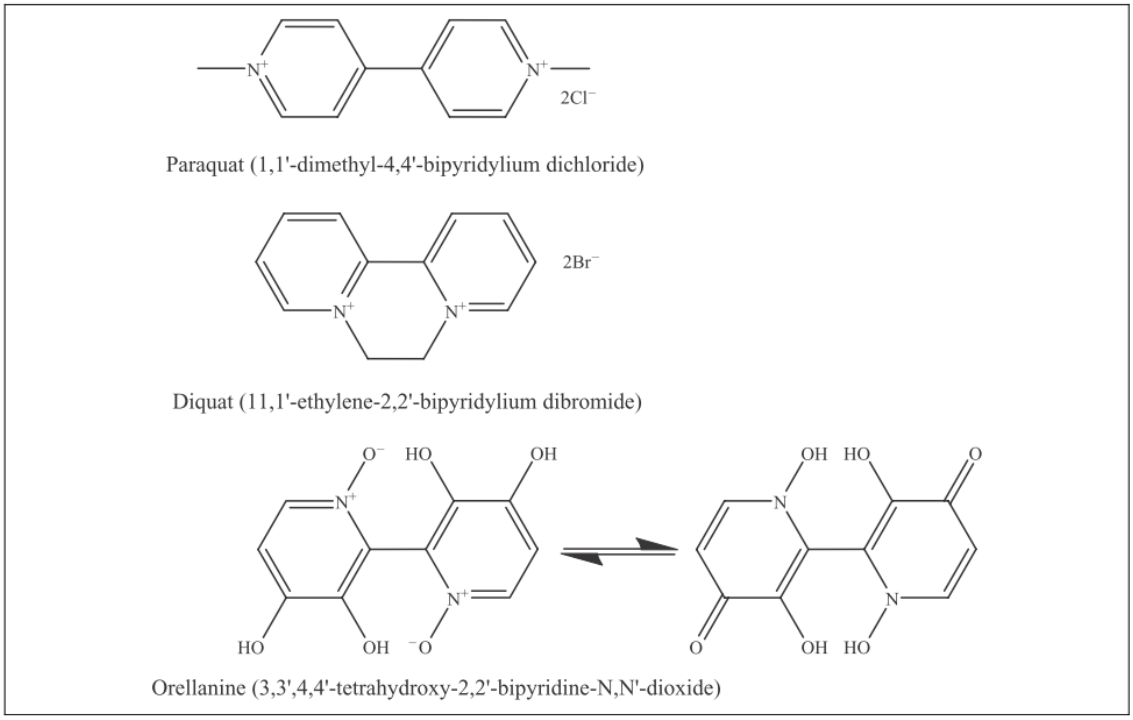

Figure 1. Chemical structures of paraquat (a), diquat (b) and orellanine (c).

Orellanine rapidly concentrates in the kidney and has been found in renal biopsy specimens up to 6 months after ingestion.[15] During the latent period, mild gastrointestinal disorders occur, which may be overlooked.[16] Accordingly, patients present themselves at the hospital only at the stage when renal failure has already developed.[17] The acute damage can be soft and brief or irreversible and even lethal. There are no specific antidotes for poisoning by *Cortinarius* species.[18] Treatment strategies include symptomatic treatment, hemodialysis, plasmapheresis, steroids, and *N*-acetylcysteine.[19] In the worst cases, only chronic hemodialysis or renal transplantation are effective.[20]

The only few reviews that have outlined the main features of this unique type of intoxication date back to the 1980s and 1990s.[10,14,21] Due to the high toxic potential and the risks to amateur mycologists, the present review article attempts to present an update of the current knowledge concerning *Cortinarius* intoxications, namely the morphological characteristics of *Cortinarius* species, epidemiological studies, chemical structure, toxicokinetics, toxic doses, mechanisms of toxicity, diagnosis, prognosis, and treatment.

Materials and methods

All articles indexed to MEDLINE® and books were searched using the following key words: orellanine, mushroom poisonings, *C. orellanus and C. rubellus*, and acute renal failure.

Cortinarius *species*

Cortinarius is the largest genus of fungi that forms mushrooms comprising over 250 species. These usually form mycorrhizae with trees and can be found in Northern Europe and in mountainous areas of Central Europe,[2] as well as in Northern America[22] at the end of summer or autumn.[22] *C. rubellus* species inhabit moist or wet conifer forest, particularly in moist acidic soil.[2]

Cortinarius species have a partial veil composed of fine threads (*cortina*) connecting the margin of the cap to the stalk, particularly in young specimens (Figure 2(a)). As the mushroom ages, the cortina disappears, resulting in almost indistinguishable remains of the cortina (Figure 2(b)).[2] They also have an orange, purple, or greenish yellow-colored fruit bodies with characteristic rusty orange gills and a

Figure 2. (a) Cortina; (b) remains of cortina; (c) *Cortinarius orellanus*; (d) *Cortinarius rubellus*; (e) *Cortinarius henrici*; (f) *Psilocybe semilanceata*; and (g) *Cantharellus cibarius*.

thick stalk with striae and bulbous base.[3] These are characterized by having a knob-like protuberance on the rust- to yellow–brown cap (2–12 cm/1–5 in) that is conical in shape on young species and more flattened on older specimens. The broad, elliptical spores yield a rust brown spore print. The cylindrical stalk is similar in color to the cap and stalk sometimes contains lemon yellow bands that represent the remains of the cortina.[2]

Specifically, *C. orellanus* (Figure 2(c)) is a medium-sized gill mushroom that is characterized by a dome-shaped, dry, orange, red, brown-to-yellow brown cap; thick, well-spaced, orange brown gills; and a dry, yellowish to reddish brown stalk that is tapered below with some veil fibrils on the surface. The body of the mushroom is whitish to yellowish. Species in this group have rust brown spore prints and the spores are elliptic and distinctly ornamented.[23] *C. rubellus* (Figure 2(d)) is characterized by a cap rust brown to orange, often has a steeper and darker colored elevation at the top of the cap, and its surface is dry and slightly scaly. The cap diameter is typically 4–8 cm when fully expanded, and the margin is often slightly rolled down even in fully mature specimens. The gills are pale yellowish at first, becoming rusty brown as the spores mature.[24] It has been claimed that *C. henrici* (Figure 2(e)) and *C. rainierensis* are identical to *C. rubellus*.[25]

Only some species of *Cortinarius* mushrooms, such as *C. orellanus*, *C. rubellus*, *C. henrici*, *C. rainierensis* and *C. bruneofulvus* contain the nephrotoxin

orellanine.[26–29] The content of orellanine was determined in dried fungus, varying greatly depending both on the mushroom portion considered and *Cortinarius* species.[30] The analysis of *C. orellanus* and *C. rubellus* species showed that the content of toxin (expressed on dry weight basis) was 9400 and 7800 mg/kg in caps, 4800 and 4200 mg/kg in stems, and 3100 and 900 mg/kg in spores, respectively. In mycorrhiza roots from *C. rubellus*, the orellanine content was 300 mg/kg.[30] In another study, the orellanine content in dried fungus was found to be about 14,000 mg/kg in *C. orellanus* and 9000 mg/kg in *C. rubellus*.[31]

Epidemiological studies

Intoxications with the fungus *C. orellanus* have been described in literature. The toxicity of the mushroom *C. orellanus* was firstly discovered in 1957 when 135 habitants of Bygdosz (Poland) were intoxicated.[7–9,32,33] Since then, most of poisoning reports have been described in Europe,[14] where mushroom foraging is popular, but cases were also described in North America and Australia.[5,14,34,35] In 1974, four cases of poisoning by *C. rubellus* occurred in Finland[36]; further incidents were reported in Scotland,[37,38] Sweden,[39] Norway,[40,41] Italy,[42] Germany,[43] France[6,44,45] and Wales, United Kingdom.[46] In Austria, Franz et al.[47] reported a *C. rubellus* poisoning on a 28-year-old man who thought had eaten magic mushrooms. Indeed, nephrotoxic *Cortinarius* species are morphologically similar to mushrooms from the *Psilocybe* genus (*Psilocybe semilanceata*; Figure 2(f)), leading to mistakes by inexperienced hallucinogenic mushrooms hunters.[47] Likewise, misidentification of *C. orellanus* as *C. cibarius* (Figure 2(g)) may also occur.[2]

Chemistry

Table 1 presents chemical properties of orellanine. It is a bipyridine *N*-oxide (2,2′-bipyridine-3,3′,4,4′-tetrahydroxy-1,1′-dioxide) that exists as two tautomers[11,48] (Figure 1). The more stable tautomer is the amine oxide form. Orellanine is a colorless fine crystalline and navy blue-fluorescing compound that is stable at 150–160°C, decomposing slowly above this temperature and under ultraviolet (UV) light to the yellow nontoxic bipyridyl compound orelline by releasing oxygen.[27] An explosive degradation occurs at temperatures over 267°C.[27] Cooking temperatures, freezing, and drying do not reduce the orellanine content.[25,49] Orellanine is stable for many years in the intact mushroom.[10] Figure 3 demonstrates the reduction of orellanine to the nontoxic compound, orelline, via the intermediate orellinine.[27,49–51] Orelline are bright yellow crystals, and orellinine is almost colorless.

Table 1. Chemical properties of orellanine.

Molecular formula	$C_{10}H_8N_2O_6$
Molar mass	252.17 g/mol
CAS number	37338-80-00
Density	1.777 g/cm^3
Boiling point	834.6°C at 760 mm Hg
Flash point	458.6°C
Enthalpy of vaporization	125.5 kJ/mol
Polar surface area	131.8 Å^2
Index of refraction	1.7
Molar refractivity	56.6 cm^3
Molar volume	141.1 cm^3
Polarizability	22.4×10^{-24} cm^3
Surface tension	86.8 dyne/cm
log *P*	−0.53 or −1.2 (depending on source)
pK_a (strongest acidic)	−9.3
pK_a (strongest basic)	−3

The stereochemistry of orellanine was confirmed by X-ray crystallography.[52] In crystal conformation, the planes of the two pyridyl rings are nearly perpendicular, making the molecule chiral. However, orellanine isolated from mushroom is an optically inactive racemic mixture, and this is probably due to the low rotational barrier to racemization.[53]

In 1962, Grzymala[32] was the first to demonstrate experimentally the nephrotoxicity of *C. orellanus* and was the first to isolate orellanine on biological analysis. Dehmlow and Schulz[54] were the first to report the successful synthesis of orellanine and orelline. Orellanine was synthesized in nine steps from 3-amino pyridine. Orelline has also been synthesized from 2-bromo-3-hydroxypyridine.[55]

As referred, when placed under UV light, orellanine is, at first, navy blue fluorescent, and after several minutes shifts to bright turquoise fluorescent due to a rapid photochemical reaction.[56] This photochemical decomposition has been shown to proceed by stepwise loss of the *N*-oxides to give first the mono *N*-oxide orellinine and, subsequently, orelline.[27]

Orellanine, orellinine, and orelline are soluble in dilute sodium hydroxide, ammonium hydroxide, and dimethyl sulfoxide; slightly soluble in methanol;

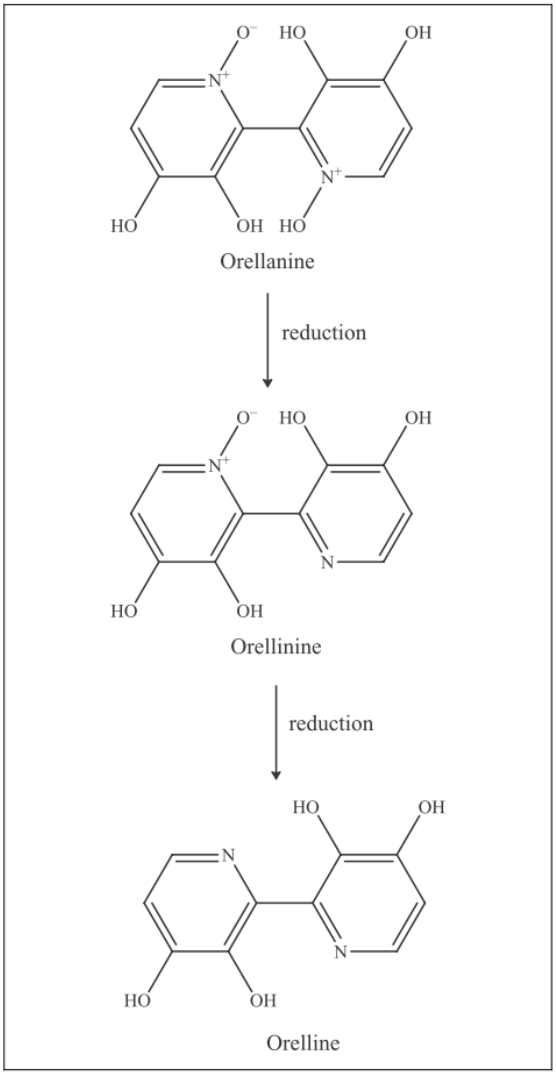

Figure 3. Chemical structures of orellanine, orellinine, and orelline.

and practically insoluble in most organic solvents and water.[10,58] Orellanine, contrary to orelline, is well soluble in alkaline solutions.

Toxicokinetics

Little is known about the toxicokinetics of orellanine in humans, especially during the first 24 h after ingestion.[57,31] In animal studies, kidneys excrete detectable quantities of orellanine only for the first 24 h after exposure.[31] This is consistent with the observations in human cases of orellanine poisoning, where the toxin is not detected in urine samples collected from 2 to 18 days after ingestion.[59,60] Typically, clinical samples are not obtained until several days after ingestion due to the delayed onset of symptoms. Indeed, this has led to misdiagnosis of the poisoning since the victim often does not associate the symptoms with a meal taken several days before. For this reason, no information is available regarding human plasma levels of orellanine attained shortly after exposure.[59]

Based on the ingestion of a toxic dose of 100–200 g of mushrooms containing 0.1% orellanine, and assuming complete absorption and full plasma distribution, maximal peak plasma levels of the toxin is expected to range from 130 to 260 µmol/L.[7,30] Based on limited testing, the clearance of orellanine from the blood is probably rapid (i.e. <2–3 days).[25]

The continued presence of orellanine in renal cortex after hemodialysis, and its persistent presence in this tissue for several months in the absence of detectable orellanine in urine or blood, suggests that the toxin is sequestered in the kidney in a poorly exchangeable form.[59]

Toxic doses

Table 2 resumes the toxic doses of orellanine and dried *Cortinarius* homogenates for different species. Several studies on the toxicity of *C. rubellus* in rats were carried out by Nieminen and collaborators.[63–65] Authors have shown that rats appear to be resistant to *Cortinarius* poisoning and a genetic variability was suggested since females were more resistant.[66] In another study,[67] 23% of the animals showed total resistance to toxins, irrespective of dose, whereas in others the observed renal lesions were dose dependent.

Clinical data indicate that humans appear to be more sensitive to toxic effects than mice and rats. The ingestion of only two to three mushrooms (3 mg of toxin that corresponds to 0.04 mg/kg for human of 70 kg) seems to be enough to make the victim dependent on dialysis for the entire life.[45,68] Similarly, in humans, the greater susceptibility of males to *Cortinarius* toxicity has been described by several authors.[14,37] Herrmann and colleagues[1] described a lethal dose of fresh *C. rubellus* between 29 g and 227 g for humans weighing about 70 kg.

Mechanism of toxicity

Orellanine and orellinine have similar toxicity, but orelline proved to be nontoxic.[69] The mechanism of

Table 2. Toxicity of orellanine and dried *Cortinarius* homogenates.

Toxin	Species	Route (ref.)	LD_{50}	LD_{100}	Remarks
Orellanine	Mouse	SC[32]	8.3 mg/kg		
		PO[61]	33 mg/kg	109 mg/kg	Male and female mice
		PO[49]	90 mg/kg		
		IP[61]	15 mg/kg	42 mg/kg	Male and female mice
		IP[49]	12.5 mg/kg		
	Cat	PO[32]	8.3 mg/kg		
	Guinea pig	IP[32]	8 mg/kg		
Cortinarius orellanus	Mouse	PO[61]	2.2 g/kg	4.57 g/kg	Containing 14 mg/g orellanine; male mice. Containing 15–20 mg/kg orellanine; female mice. Containing 15–20 mg/g orellanine; male rats.
		PO[49]	2.1 g/kg		
	Rat	PO[31]	0.976 g/kg		
Cortinarius rubellus	Mouse	PO[61]	3.12 g/kg	6.22 g/kg	Containing 9 mg/g orellanine; male mice

SC: subcutaneous; PO: *per os*; IP: intraperitoneal; LD_{50}: median lethal dose.
Source: Adapted from the work by Carter et al.[62]

toxicity is not yet fully understood. It was shown that the toxin inhibits the synthesis of macromolecules such as proteins, RNA, and DNA[14,25,70]; promotes a noncompetitive inhibition of the activity of alkaline phosphatase, γ-glutamyl transpeptidase, and leucine aminopeptidase[71]; and interrupts the production of adenosine triphosphatase,[69] namely at the proximal tubular brush border, compromising utilization and reabsorption of peptides, polysaccharides, and other molecules.

Moser[72] showed that *C. orellanus* toxin inhibits DNA-dependent activity of RNA polymerase B from rat liver (eukaryotic cell) and DNA-dependent activity of RNA polymerase from *Escherichia coli*. Later, Richard and co authors[70] pretreated rat liver microsomes with orellanine and then exposed rabbit reticulocyte lysate to this mixture. Only the inhibition of protein synthesis was observed, while direct addition of untreated orellanine or only microsomes did not. This suggests that the inhibition of protein synthesis is due to a metabolite of orellanine.

Orellanine inhibits pinocytosis in *Amoeba proteus* and inhibits growing of both slime mold *Discyostelium discoideu* and *E. coli* at 80 μM.[69] The large spectrum of toxic effects in plants, animals, and microorganism suggests that the target is likely to be a cellular process found in both prokaryotes and eukaryotes.[49]

Rapior et al.[29] suggested that the *Cortinarius* toxicity is caused by metabolites with the isoxazolium core derived from the photochemical rearrangement of orellanine (called the phototoxicity mechanism of orellanine). In accordance, authors found that orellanine purified in the dark and administered to laboratory animals show low toxicity, while the one extracted in the light induces a toxic response (Figure 4). These intermediates can bind covalently with numerous proteins in the body, leading to organ damage.

As already referred, orellanine chemically resembles the pyridine herbicides diquat (1,1′-ethylene-2,2′-bipyridinium) and paraquat (1,1′-dimethyl-4,4′-bipyridinium), and some authors suggested a possible mono-electronic reduction mechanism (generation of a stable radical) for its toxicity mechanism.[10,13] The consequence is obvious: production of free radicals and therefore oxidative stress similarly to paraquat.[73,74] In opposite to paraquat, this proposed intracellular toxic mechanism with nicotinamide adenine dinucleotide phosphate plus H^+ depletion would need a long time to damage the cells to the degree of inevitable necrosis, according with the delayed toxic effects observed in this type of mushroom poisoning.[75]

However, this model has been criticized since orellanine has a much more negative redox potential than paraquat and diquat.[20,76] Noteworthy, these authors observed the formation of a radical form of orellanine induced by near UV (370 nm) at physiological pH under aerobic or anaerobic conditions.[20] This apparently stable radical, identified as *ortho*-semiquinone anion radical (Figure 5), was also generated using biological oxidizing agents (e.g. cytochrome *c* and nicotinamide adenine dinucleotide) or enzymatic systems such as tyrosinase/oxygen (O_2) and peroxidase/hydrogen peroxide (H_2O_2).[13,50] Indeed, it is the *ortho*-semiquinone anion radical that is responsible for superoxide radical production. At the renal spot

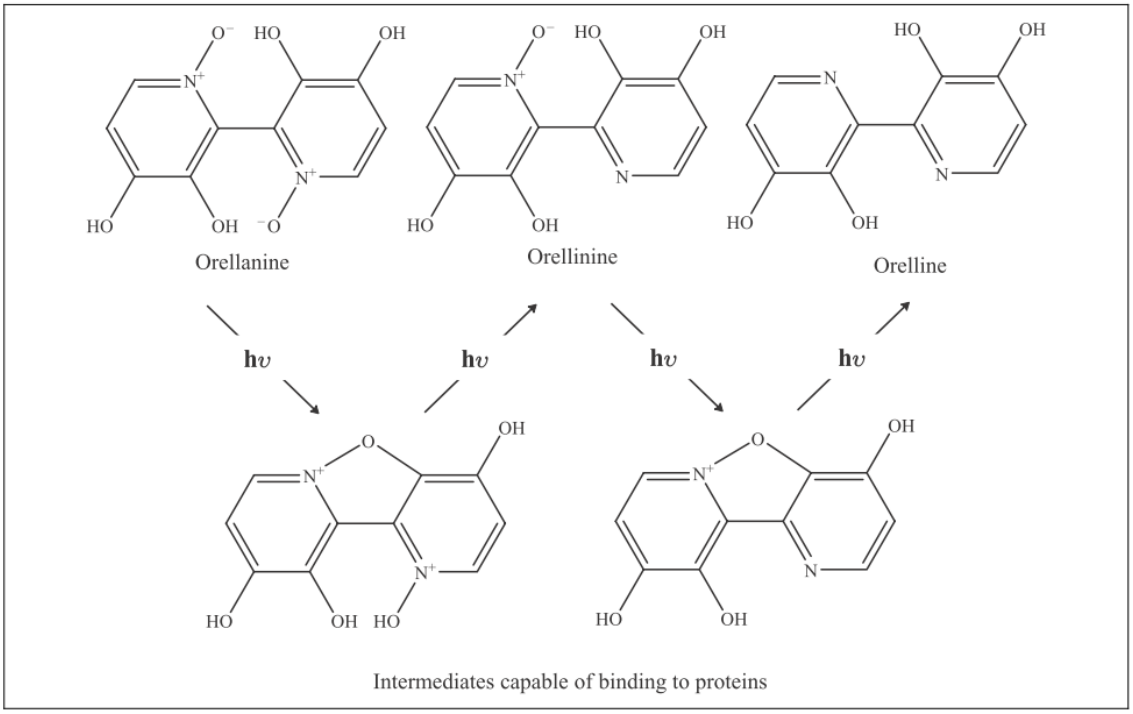

Figure 4. Phototoxicity mechanism of orellanine.

of bioaccumulation of the toxin, a quick change of small portions of orellanine rapidly cycling from its oxidized to its reduced form might be sufficient to support continuous production of reactive oxygen species, which will induce oxidative stress. This process may lead to a large oxygen consumption, which might create hypoxic conditions, as well as the dramatic depletion of renal glutathione and ascorbate levels consequently making cells more susceptible to oxidant damage.[12,13,50] Corroborating this hypothesis, orellanine-treated animals did experience increased oxidative stress, which is indicated by increase in both plasma levels of ascorbyl radicals and protein oxidation in renal tissue.[11]

Oubrahim and colleagues[28] proved the formation of a characteristic *ortho*-semiquinone radical anion ($SQ^{\cdot}$) derived from orellanine in a system containing horseradish peroxidase and H_2O_2. Since peroxidative oxidation proceeds via two one-electron oxidation steps, this enzyme can oxidize two molecules of orellanine to the corresponding semiquinone during one enzymatic cycle or one molecule of orellanine to the corresponding quinone by two successive monoelectronic oxidation steps. The *ortho*-semiquinone form of orellanine was also generated by the enzymatic system tyrosinase/O_2, which proceeds via a two-electron oxidation. In this case, the generation of the *ortho*-semiquinone radical likely occurs due to the equilibrium between orellanine and its corresponding quinone. During oxidation of orellanine in renal tissue, *ortho*-semiquinone likely accumulates, whatever the mode of enzymatic oxidation may be (one or two electrons). The *ortho*-semiquinone, as well as the quinone, could participate in a variety of reactions including covalent binding to biological compounds leading to cell damage. In addition, when oxidation of orellanine by horseradish peroxidase/H_2O_2 occurred in the presence of reducing agents of biological interest, such as ascorbic acid or glutathione, the formation of ascorbyl or glutathionyl radicals was observed.

Although all authors agree that orellanine is the main toxin, *Cortinarius* species also contain cyclic decapeptides (cortinarin A, B, and C) that produce renal damage.[77,78] Cortinarin A and B (not C) were found to be nephrotoxic in animal studies,[78] but the role of these decapeptides in causing the Orellanus syndrome or even their existence has been questioned by several authors.[79]

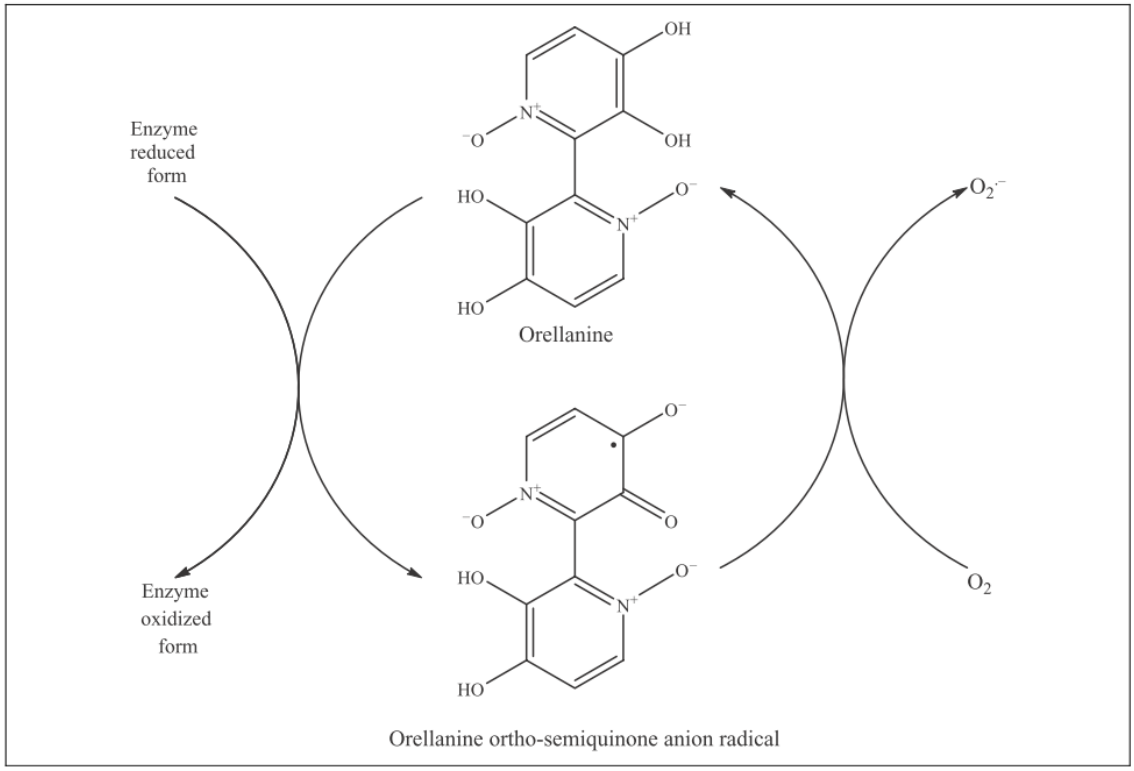

Figure 5. Redox cycling of orellanine.

Diagnosis

The diagnosis of orellanine poisoning can be clinical, mycological, or toxicological.[1,3,51]

Clinical diagnosis. Orellanine poisoning is characterized by a long latent period. Typically, the onset of symptoms is delayed from 2–4 to 14 days after ingestion.[14] The higher the quantity of mushrooms consumed, the shorter the latent period.[62] There is a significant variation on individual responses to poisoning, with the degree of renal injury ranging from mild and transient to severe and irreversible.[59]

Some patients suffer mild gastrointestinal discomfort in the latency period before developing signs of renal impairment. Table 3 shows signs, symptoms, and analytical findings related to orellanine poisoning. The gastrointestinal phase (also known as prerenal phase) is primarily characterized by vomiting and nausea, and, less often, abdominal pain, asthenia, chills, burning sensation in the mouth, thirst, headache, myalgia, anorexia, and diarrhea.[10,14,15,21,25]

Clinical signs may spontaneously disappear in some patients, leaving the poisoning unnoticed; in others, the signs become more intense and are accompanied by neurological manifestations (e.g. paresthesia, taste impairment, cognitive disorders, and dizziness), lumbar pain, and anuria requiring hospitalization.[14,15,21]

Renal failure may develop several days to weeks after initial symptoms due to orellanine sequestration in the kidney. The incidence of renal failure varies from 30% to 70% of patients, and 30–65% need temporary dialysis.[14] Renal involvement is the other peculiarity of this poisoning and is mainly characterized by lumbar and flank pains, intense thirst, oliguria, polyuria, proteinuria, hematuria, and leukocyturia.[10,14,21,23,80] The blood balance shows an increase in urea and creatinine levels.[21,23] Differential diagnosis with other nephrotoxic compounds such as oxalates crystals found in certain plants (e.g. genus *Philodendron* and *Dieffenbachia*) and ethylenoglycol found in antifreeze products is relatively easy. In these poisonings, signs and symptoms are evident

Table 3. Signs, symptoms, and analytical finding in intoxications by orellanine.

Prerenal phase
Vomiting, polydipsia, lumbar pain, nausea, vomiting, abdominal pain, headache, polyuria, asthenia, diarrhea, anorexia, myalgia, faintness, parestesia, constipation, chills, somnolence, vertigo, dysgeusia, sweats, tinnitus, burning in mouth, fatigue, thirst, dry mouth, visual defects, loin pain, and paresthesia
Renal phase
Myalgias, intense lumbar pain, flank pain, oliguria, leukocyturia, hematuria, proteinuria, anuria, glucosuria, leucocytosis, increased serum creatinine, potassium and urea, renal histopathological analysis evidencing tubule-interstitial nephritis, interstitial edema, inflammatory infiltrates, and fibrosis/sclerosis
Treatment
Hemodialysis/peritoneal dialysis, extracorporeal hemoperfusion, plasmapheresis, corticosteroids, diltiazem, dopamine, selenium *N*-acetylcysteine, and renal transplant

during the first hours after exposure and circumstantial evidences can help diagnosis.[81]

Nephrotoxicity is characterized by an early and severe interstitial fibrosis, interstitial edema, and tubular epithelial necrosis.[10,21,51,80] Short and colleagues[37] have shown that when initial biopsy was made until 2–3 weeks after poisonings, specimens exhibited pronounced focal tubular damage with tubulorrhexis, cast formation, and severe interstitial edema with patchy infiltration of lymphocytes, plasma cells, and some polymorphs. The glomeruli showed only slight mesangial cell reaction. In the later biopsy specimens (obtained 7–8 weeks after poisoning), the mild glomerular reaction was still present, but the major features were tubular dilatation and cellular atrophy with groups of apparently normal tubules between damaged areas. In both cases, the interstitial edema was much less in the later specimens, but there was early fibrosis between the damaged tubules. A mild mononuclear inflammatory cell infiltration was present. Immunofluorescence studies showed no significant deposition of immunoglobulin, complement, or fibrin in glomeruli, but immunoglobulin G, immunoglobulin A, and fibrin were isolated from tubular casts.[37] Therefore, the existence of renal alterations as a consequence of some immune reaction cannot be excluded.

In poisoning due to *C. rubellus*, most cases have not been admitted to hospital before 8–14 days after the mushroom ingestion and then the clinical picture is that of an acute renal failure.[10] It is estimated that 30–45% of individuals who ingest nephrotoxic *Cortinarius* mushrooms develop acute renal failure.[14] Of these, half usually recover the renal function and half progress to chronic renal insufficiency and require maintenance hemodialysis or kidney transplant.[59] Some patients may become asymptomatic and the renal injury is only identified by biochemical tests.[46] In a study of 26 patients with nephrotoxicity secondary to *Cortinarius* mushroom ingestion, the incidence of end-stage renal failure requiring dialysis and renal transplantation was approximately 8%,[6] whereas 9 of 22 (41%) Swedish patients developed end-stage renal disease after ingesting mushrooms from *Cortinarius species*.[15]

Liver injury has also been observed based on increase of transaminases and bilirubin levels, hepatomegaly, hepatalgia, and from lipoidosis and necrosis lesions evidenced by histological analysis,[8,33] but most of the studies ruled out liver involvement.[14]

Worth mentioning, orellanine is currently being tested as a potential treatment for metastatic renal cancer based on its highly selective toxicity to renal cells.[82]

Mycological and toxicological analyses. Several analytical methods were developed for the analysis of both mushrooms and biological samples, such as serum and renal tissue. The ferric–orellanine reaction could be useful for both mycologists and medical personnel for demonstrating whether an unknown *Cortinarius* specimen contains orellanine or not. Schumacher and Hoiland,[10] have proposed a rapid qualitative test to detect orellanine in mushrooms. A fresh or dried mushroom is crushed in five volumes of water and filtered after 10 min at room temperature. The filtrate is then mixed with an equal amount of 3% ferric chloride hexahydrate dissolved in 0.5 N hydrochloric acid (HCl). The presence of orellanine is suspected if a dark gray–blue color ink appears. However, other authors described an immediate change of the yellowish liquid to purple–red or violet.[83]

Thin-layer chromatography can be used for separation prior to proof for the presence of toxins under UV light. A simplified procedure for detecting orellanine is as follows: a small piece of dried fungus is crushed

and extracted in 50% ethanol, allowed to stand for 15 min at room temperature, and then the extract is applied to a silica gel plate and chromatographically developed by *n*-butanol:acetic acid:water (3:1:1). After drying, the plate is sprayed with 2% ferric chloride in 0.5 N HCl. Orellanine is visible as navy blue spot from the application point (retention factor of 0.25–0.5), orellinine as dark blue spot, and orelline as light blue spot.[10,22] Other analytical procedures are available such as electrophoresis[28] and high-performance liquid chromatography (HPLC) with photodiode array,[30] electrochemical,[84] and UV[85] detection. In these last two methods, reversed-phase ion-pair HPLC with phosphate-containing eluent20 and amide and C18 columns with phosphoric acid as the eluent10 were employed. Orellanine has also been detected in single MS mode using electron impact[84] and electrospray ionization.[1,30]

In a renal biopsy specimen, orellanine can be detected up to 6 months after poisoning by performing a thin-layer chromatography technique.[51,60,86] Orelline, the di-reduction metabolite, has also been found in renal biopsies in cases of orellanine poisoning and possibly originates from either the mushroom itself or from extra- or intrarenal metabolism of orellanine.[59]

Since orellanine is rapidly concentrated in the kidney, it will not be detected in urine, blood, and dialysis fluids at the time when the first symptoms appear.[87] Toxin in plasma can be only detected for up to 2 weeks after ingestion.[53,86] However, Andary and colleagues[86] were able to detect a plasma orellanine concentration of 20 μmol/L in a sample obtained from a patient 9 days after eating two mushrooms, and they used hemodialysis to effectively clear the circulating toxin. The toxin is not detected in urine samples collected as early as 2 days after ingestion and as late as 18 days.[51]

Light microscopy has also been useful to highlight characteristic renal histopathological findings such as acute tubular injury and interstitial edema and invasion of inflammatory cells with interstitial nephritis.[87]

Prognosis

The chance of full recovery from orellanine poisoning depends on the amount of ingested toxin, age, and general health of the individual patient, probably preexisting subclinical renal disease, and the time at which therapy is started.[22]

Holdmdalh[88] proposed a method for estimating the prognosis in individual cases. This method is called the "*Cortinarius* nephron toxicity prognostic index (CNT)" and is based on two parameters generally available. The parameters used are the serum creatinine level before treatment and the former days after ingestion of the mushrooms. It is calculated from the formula $CNT = (y + 316)/X \times 10^4$, where y is the serum creatinine and X the number of elapsed days. CNT index <1.1 indicates a good prognosis; CNT between 1.1 and 2.1 indicates "intermediate" prognosis (probably chronic renal failure with serum creatinine higher than 200 μmol/L) and >2.1 a poor outcome with end-stage renal failure requiring renal replacement therapy.

Grzymala[8,33] established a relationship between the duration of the latent period and poisoning severity. In his series, the latent period was:

a. 10–17 days in patients presenting with thirst, burning sensation in the mouth, and polyuria (mild intoxication);
b. 6–10 days in patients presenting with digestive disorders, polyuria or oliguria, hematuria, and leucocyturia, but no significant renal impairment;
c. 2–3 days in patients with acute renal failure and death rate of about 50%.

Treatment

There is no specific antidote to orellanine poisoning, treatment being mainly supportive care as well as the use of hemodialysis as needed. Prolonged monitoring of renal function is necessary because of the slow resolution of kidney dysfunction.[2]

Emesis or gastric lavage might, in theory, be indicated if the patient is seen earlier than 6 h after ingestion.[69]

Extracorporeal hemoperfusion, hemodialysis, and plasmapheresis are techniques used to remove the toxin from circulation but should only be considered if the patient is seen within 1 week after ingestion.[22,42,51,89–91] Beyond that period, the use of hemodialysis depends only on the need to support renal function.[2] About half of the patients requiring dialysis did not recover kidney function.[14]

There are also reports of cases where the use of corticosteroids, *N*-acetylcysteine, and selenium allowed clinical improvement.[3,90] *N*-Acetylcysteine is a glutathione donor and antioxidant,[13] and selenium is an essential component of several major metabolic pathways including immune and antioxidant defense systems.[92]

It has been emphasized that forced diuresis could rather accelerate and amplify the nephrotoxic process since it possibly accentuates the accumulation of the toxins in the kidney and therefore it is not recommended.[10,69]

Complete recovery of renal function is attained only in 30% of the poisoned patients with the majority of patients healing with fibrosis and a variable loss of renal function, and 20–40% patients requiring long-term renal replacement therapy.[6,90] Therefore, renal transplantation has been considered, but should not be performed too early in the course of illness. The median time for transplant is 9–10 months after presentation, wherein appears safe without risk of further toxicity from orellanine.[14,15]

Concluding remarks

The incidence of mushroom poisoning greatly varies all over the world depending on local traditions, lifestyle, nutritional factors, climate, and the occurrence of wild mushrooms. Orellanine poisoning is mostly accidental and the result of a mix-up between edible and toxic fungi. Only by exception, mushroom poisoning has a suicidal or homicidal cause. Confusion with many nephrotoxic *Cortinarius* species occurs every year throughout Europe and North America. This poisoning is associated with high morbidity and exceptional mortality. Treatment of orellanine poisoning includes gastrointestinal decontamination and general symptomatic and supportive care. Specific antidotes do not exist. Further knowledge of orellanine toxicokinetics and toxicity mechanisms is still necessary to be able to propose a proper treatment.

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